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FRACTIONAL SOLUBILITY OF GLUTEN IN SODIUM SALICYLATE SOLUTIONS[1]

BY E. Y. SPENCER[2] AND A. G. MCCALLA[3]

Abstract

The amount of gluten protein dispersed by sodium salicylate solution is proportional to the concentration of the salt, while the completeness of extraction with any concentration depends on the penetration of the salicylate solution into the gluten particles. The higher concentrations (5 to 10%) penetrate rapidly because they disperse most of the protein and automatically expose fresh surface for attack. The lower concentrations penetrate slowly unless the non-dispersed protein is occasionally removed by vigorous stirring or shaking. The amount of protein in dispersed form in any specific concentration of salicylate is the same whether determined by extraction or by dilution of a dispersion in 8% salicylate.

The amide nitrogen of protein fractions obtained by fractional solubility is the same as that of corresponding fractions obtained by precipitation. Results confirm an earlier conclusion that, except for the most soluble 15%, gluten protein consists of a single complex that can be progressively fractionated.

Introduction

Studies on the fractionation of gluten dispersed in sodium salicylate solutions (12) led to the conclusion that the major part of the protein in gluten forms a single complex that can be reversibly fractionated into an indefinite number of component parts differing systematically in physical and chemical properties. Since these results were published there have appeared several papers (2–5) in which the authors reach conclusions at variance with this. The most important of these from our point of view is that by Blish (2), in which the results of extensive experiments employing both fractional precipitation and fractional solubility methods are discussed. The results obtained using the former supported the conclusions of McCalla and Rose, while those obtained using the latter did not. An independent consideration of Blish's results is impossible as no experimental data were published. It is sufficient at this point to note that Blish favors the hypothesis that flour proteins are constituted of relatively few components or component groups.

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[2] *Graduate Assistant.*

[3] *Research Assistant, Associate Committee on Grain Research, University of Alberta, Edmonton.*

He considers that fractional precipitation methods have not furnished trustworthy and conclusive evidence regarding the number of protein fractions. Further discussion of these conclusions is only possible after presentation of the results of the present study.

Harris and Bailey (5) and Harris (3, 4) also favor the hypothesis of few protein components, although the published results have all been obtained from studies in which the isolation of relatively few fractions was attempted. They state that alterations in the conditions of fractionation resulted in different proportions of the three groups obtained. This in itself is definite evidence against the individuality of the fractions obtained, but since fractional precipitation methods were used, these results are open to the general criticisms outlined by Blish.

Harris used the method of preparing and fractionating dispersions in sodium salicylate as outlined by McCalla and Rose (12). He encountered difficulty in securing concordant replicate results when magnesium sulphate solution was added to the dispersions. Since Harris was primarily interested in the relations existing between the quantities of the various fractions and wheat quality, this study yielded little new information regarding the fundamental nature of the protein. Nevertheless Harris is inclined to believe that the fractions obtained are thrown down as mixtures containing varying proportions of each of the few components.

Rich (13) made a study of the dispersion of flour protein in various salt solutions. His results support the conclusion that there are no clear-cut borders in the solubility of flour proteins.

Further evidence has been presented in support of the conclusion that so-called "gliadin" is itself a component system. Krejci and Svedberg (7) found by means of ultracentrifugal determinations that gliadin as extracted by 64% alcohol is heterogeneous as to molecular weight. The least soluble fraction contained a high concentration of heavy molecules, while the most soluble fraction consisted almost entirely of the constituent of lowest molecular weight. Lamm and Polson (10) studied similar gliadin fractions by means of diffusion constants obtained with a refractometric method. All but the most soluble fraction proved to be non-uniform. Kuhlmann (9) studied gluten using alcohol solutions of various concentrations, and concluded that gliadin is not a chemical individual. He suggests that the length of the micelles of the various gliadin components vary, and that glutenin is made up of micelles which are longer than those found in gliadin.

Krejci and Svedberg (8) have further demonstrated that the protein fractions extracted from wheat flour with each of the halides of potassium are mixtures of proteins, and that the mean molecular weight increases from that of the mixture extracted with the fluoride to that of the mixture extracted with the iodide.

The results of a study (11) made on the nitrogen of developing wheat grain support the protein complex hypothesis. These results indicated that the non-protein nitrogen present in the wheat kernel at any definite stage of devel-

opment was the precursor of a specific fraction of the gluten protein. The regularity of the changes in composition of the non-protein nitrogen fraction, and the subsequent formation of corresponding protein fractions make it appear unlikely that there would be any sharp break in the properties of the successive gluten protein fractions.

The work discussed in this paper was undertaken with the object of determining the validity of the conclusions reached as a result of fractional precipitation studies. It was recognized that Blish's criticisms of fractional precipitation methods were justified if there were at least three protein components in gluten. The results presented in this paper were therefore obtained using fractional solubility methods.

Materials and Methods

All experiments reported in this paper were carried out using an unbleached flour milled from high quality, sound Reward wheat.

Gluten was washed from the flour and dispersed in essentially the manner described by McCalla and Rose (12). The principal change in the procedure finally adopted was the introduction of a mechanical stirrer, instead of shaking by hand the flasks containing gluten and solution. The ball of washed gluten was divided into pieces approximately 3 to 5 mm. in diameter, placed in 200 cc. of solution in a 500-cc. wide-mouthed Erlenmeyer flask, and stirred for five minutes.* The flask was set aside at room temperature for 22 hr., and then the contents were stirred for 15 min. The non-dispersed fraction was removed by centrifuging at 2500 r.p.m. for 20 min. The liquid was decanted, and the non-dispersed protein washed twice with sodium salicylate solution of the same concentration as that used in dispersion. When gluten was re-extracted with a second lot of solution, the procedure was repeated.

Concentrations of sodium salicylate from 0 to 10% were used, and all solutions were saturated with toluene. This was essential to prevent biological activity in water, and was used throughout for the sake of uniformity. In all but one experiment, the salicylate used was from a single lot.

Total nitrogen was determined by the Kjeldahl method, using mercuric oxide as catalyst. Hydrolysis and analysis of the gluten fractions followed the methods used in the earlier work (12).

Results

Fractional solubility of gluten

The amount of gluten nitrogen dispersed by sodium salicylate solutions had been found to be roughly proportional to the concentration of the solution (12). As it seemed possible that in the lower salicylate concentrations the extraction of dispersible nitrogen was incomplete, this experiment using four concentrations of sodium salicylate was repeated. Instead of a single extraction being made, each gluten sample was extracted four times, the dispersed

* *Since this study was made, it has been found that it is better to omit the initial stirring. As dispersion depends on penetration of the gluten by the solution, little or no dispersion takes place when the gluten particles are stirred immediately after being placed in the salicylate.*

protein being removed after each extraction. The results are shown in Table I, each figure being the mean of duplicate results. The duplicate values given are for parallel experiments conducted several weeks apart.

TABLE I

PERCENTAGE OF TOTAL GLUTEN NITROGEN DISPERSED BY FOUR SUCCESSIVE EXTRACTIONS WITH SODIUM SALICYLATE

Extraction No.	Concentration of sodium salicylate, %							
	0		2		5		8	
	a	b	a	b	a	b	a	b
1	5.5	5.7	34.1	38.5	70.7	70.1	96.8	96.5
2	7.0	3.5	6.7	6.8	3.2	4.2	0.5	0.8
3	2.5	2.0	4.0	2.6	1.1	1.2	0.3	0.2
4	3.6	1.8	2.1	1.7	0.6	0.6	0.1	0.0
Total	18.6	13.0	46.9	49.6	75.6	76.1	97.7	97.5

There is good agreement between the results for the two experiments except where water was used as the dispersing agent. The first extraction was most effective with the 8% sodium salicylate, and progressively less effective as the concentration decreased. While negligible amounts were dispersed by the fourth extraction with 8 and 5% salicylate, it seemed evident that there was still incomplete dispersion by 2% and water. The agreement between the results of the two experiments with water was relatively poor. The reason for this is discussed later.

The effect of 2% sodium salicylate over a more prolonged extraction period was then investigated. Samples of gluten were extracted for nine successive days and the dispersed nitrogen was removed after each extraction. The results are given in Fig. 1. These demonstrate conclusively that the dispersed nitrogen approaches a limit, which in this experiment was approximately 52% of the total gluten nitrogen. At the same time a sample of gluten was dispersed in 8% sodium salicylate, and the dispersion diluted to 2% salicylate. The amount of nitrogen remaining in dispersed form was 51% of the total, which is in excellent agreement with the total dispersed with repeated extraction of the gluten.

As it had been found that repeated extraction was necessary to effect complete removal of the gluten nitrogen dispersible by the lower concentrations of salicylate, and that the amount of nitrogen so dispersed in 2% salicylate was the same as the amount remaining dispersed after dilution of the salicylate from 8 to 2%, it was decided to compare the values obtained using the two methods over the whole range of unit concentrations from 1 to 8%. Dispersions were stirred for four successive days, but the dispersed material was not removed until after the fourth day. Preliminary tests had shown that it was the exposure of fresh surface, and not the removal of dispersed nitrogen,

that led to increased dispersion with repeated extraction. At the same time a stock dispersion in 8% sodium salicylate was prepared. Aliquots of this were diluted to give the desired concentration of sodium salicylate, and to give the same concentration of nitrogen as in the individual dispersions.

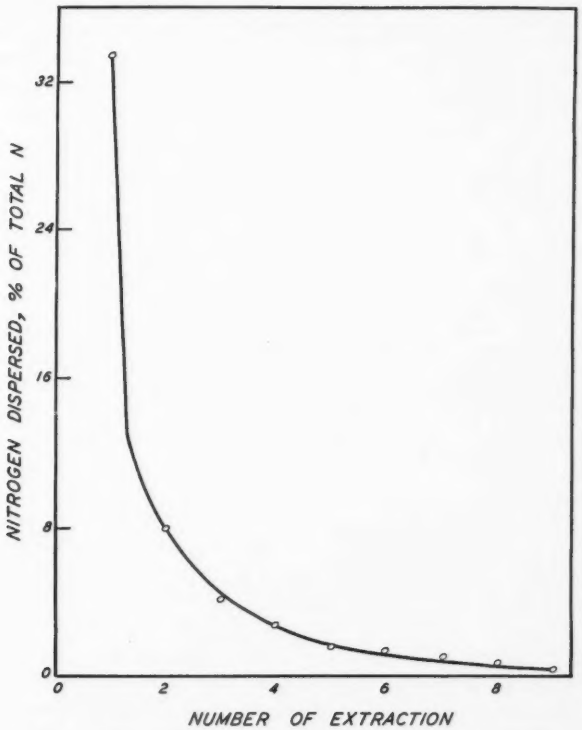

FIG. 1. *Nitrogen dispersed on repeated extraction of gluten with 2% sodium salicylate.*

Dispersed and non-dispersed fractions were separated by centrifuging. The results are given in Fig. 2. There is excellent agreement over the whole range of concentration, with the amounts obtained by dilution being slightly higher than those obtained by dispersion. This was to be expected, in the lower concentrations at least, as dispersion is not quite complete even after four successive extractions.

The amounts of nitrogen dispersed by the two methods are much lower than for the corresponding concentrations of sodium salicylate in Table I. All the earlier experiments had been carried out with C.P. grade salicylate, but in this particular experiment U.S.P. grade was used. As this offered

the only reasonable explanation for the differences, the experiment was repeated with a new lot of C. P. grade salicylate in concentrations of 2, 5 and 8%. The results are given in Table II and Fig. 2. These values are considerably higher than for the U.S.P. salicylate, but lower for the 2 and 5% concentrations than those in Table I. Investigation showed that the differences in dispersing power were related to differences in pH of the salicylate solutions, and to salt impurities. This phase of the problem is at present under investigation, as the standardization of salicylate solutions is essential if concordant results are to be obtained.

TABLE II

PERCENTAGE OF TOTAL GLUTEN NITROGEN DISPERSED BY FOUR SUCCESSIVE EXTRACTIONS, AND REMAINING DISPERSED AFTER DILUTION FROM A CONCENTRATED SOLUTION, C.P. SALICYLATE

Method	Concentration of sodium salicylate, %		
	2	5	8
Dispersion	36.7	69.6	97.6
Dilution	40.3	71.3	97.8

TABLE III

PERCENTAGE OF TOTAL GLUTEN NITROGEN DISPERSED BY SODIUM SALICYLATE AFTER EXPOSURE FOR DIFFERENT LENGTHS OF TIME

Time of exposure, days	Concentration of sodium salicylate, %			
	0	2	5	8
1	5.7	33.5	70.1	96.7
3	15.3	34.7	71.3	96.2
6	35.4	36.1	73.5	95.7
9	41.7	37.3	75.3	97.5

That time of exposure to sodium salicylate solutions is not the main factor in the dispersion of gluten nitrogen is shown by the results in Table III. Glutens were prepared as usual, but after the initial stirring were set aside for periods of 1, 3, 6 and 9 days. Dispersion in 8% solution was almost complete at the end of one day, a result in agreement with those in Table I. Dispersion in 5% was slightly less at the end of nine days than it was after four successive extractions, although the gluten had been exposed for more than twice as long. This effect was much more marked with the samples in 2% salicylate. The amount of nitrogen dispersed after nine days' exposure *without stirring*, except at the end of the exposure, was 10% less than after four successive extractions, and about 15% less than the total amount extractable with 2% salicylate. Thus the removal of the non-dispersed gluten and the exposure of fresh surface is required before complete extraction of the specific protein fractions soluble in the lower salicylate concentrations can be secured.

The behavior of the gluten in water was abnormal. Although the water was saturated with toluene, some factor other than the normal dispersing action was effective in increasing the amount of nitrogen dispersed. This factor appeared to be inhibited in sodium salicylate solutions. It seems likely that the effect was brought about by a disaggregating enzyme of the type discussed by Blagoveschenski and Yurgenson (1). This is indicated

by the results of analyses of the protein dispersed. When only a short extraction period is used, the water-soluble protein is low in amide and high in arginine nitrogen. With extraction periods such as were used in this experiment, however, the amide and arginine percentages approach those of the whole gluten, indicating that there is disaggregation of the gluten as a whole. This phase of the work has not been continued, but obviously water should not be used as a dispersing agent for gluten if the experiment is to be carried over any appreciable period of time.

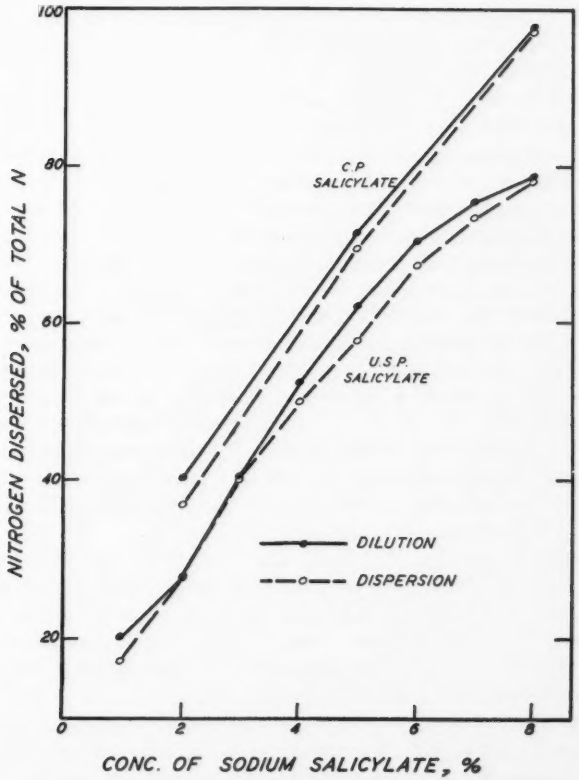

FIG. 2. *Comparison of the amount of gluten dispersed by repeated extraction, and by dilution of a complete dispersion in 8% sodium salicylate.*

The results of extraction and precipitation experiments support the hypothesis that gluten protein is made of a large number of components. In the fractional precipitation studies, however, individual fractions representing only small portions of the protein had been isolated and analyzed.

Attempts to obtain similar small but definite fractions by solubility methods met with comparatively little success. The results, however, are important in the consideration of the methods and of the apparent discrepancies between results obtained with them and with precipitation methods (2). Briefly summarized, these results are as follows:

If gluten is extracted a sufficient number of times to remove most of a fraction of specific solubility, considerable denaturation of the remaining protein results. This is shown by the fact that four extractions of gluten with 1% and re-extraction with 2% sodium salicylate failed to remove as much total protein as a single extraction with the 2% salicylate. Likewise when gluten was extracted four times with 1% salicylate, complete dispersion of the remainder in 10% could not be accomplished. The amount remaining undispersed was 14.5%, although after a single extraction of gluten with 10% salicylate, only 2 to 3% remained undispersed.

The mechanical stirring of the gluten and solution appears to be the cause of the denaturation. Continuous stirring or shaking under similar conditions dispersed less protein than did a single extraction as used in this study. Thus there are two opposing factors effective in determining the amount of protein that can be dispersed in any specific concentration of salicylate. It is necessary to use such mechanical means as stirring or shaking in order to remove the non-dispersed fraction from the unextracted portions of the gluten, but the greater the mechanical agitation, the greater the denaturation. The critical factor in the dispersion of any specific gluten fraction is the penetration of the gluten by the salicylate solution. The higher concentrations of sodium salicylate penetrate rapidly because they disperse most of the protein and hence automatically expose fresh surface. Probably nearly complete extraction of any specific protein fraction could be effected in much less time than is now necessary, if the right combination of frequency and degree of agitation could be determined.

Finally, this denaturation results in overlapping of solubilities of otherwise distinct fractions. Part of the denatured protein is removed when the gluten is re-extracted with a higher concentration of salicylate, and contaminates the main portion of this fraction. How important this factor may have been in Blish's (2) attempts to isolate individual fractions with definite properties it is impossible to say, but it certainly cannot be ignored.

The results obtained by Rich (13) were so contrary to those discussed here that his experiment with sodium salicylate was repeated. He reported that 4% ($N/4$) salicylate removed 85% of the protein from flour with one extraction and 100% with two. Our results show only 63% dispersed with two extractions. This is a smaller proportion of the gluten nitrogen than is dispersed when washed gluten is used. Furthermore, 8% salicylate dispersed only 75% of the flour nitrogen and caused marked gelatinization of the starch. The only explanation that can be offered for the results obtained by Rich is that he apparently did not use a centrifuge, and so his "dispersion" may have been, to a considerable extent, a suspension. This is not unlikely, as gluten

protein that appears to be completely dispersed may yield considerable solid matter when centrifuged in an ordinary centrifuge, and still more when a supercentrifuge is used. Differences in pH of different salicylate solutions could not explain the results obtained.

Analysis of fractions

As the work on extraction had shown that definite individual fractions of the gluten protein were difficult to isolate by solubility methods, it was not surprising to find that the results of amide analyses of these fractions failed to show the variation exhibited by the fractions obtained by precipitation. There were many factors that combined to render these results unreliable, however, so other methods of obtaining fractions for chemical analysis were sought.

The extraction of individual samples of gluten by various concentrations of sodium salicylate had yielded results showing systematic variation in the gluten from the most to the least soluble fractions. By selecting a suitable concentration of salicylate, the gluten could be separated into dispersed and non-dispersed portions at any desired point. These dispersed and non-dispersed portions appeared to be the best material for use in amide analyses, since any differences in amide content of the various portions must be the result of differences in amide content of the fractions removed by progressively more concentrated salicylate. The "fractions" thus obtained could not be formed by the type of adsorption discussed by Blish, as no fractional precipitation was employed.

Eight gluten balls were prepared from 10-gm. flour samples. Two balls were broken up and extracted four times with 2%, two balls with 3%, two with 5%, and two with 8% sodium salicylate solutions. The protein not dispersed was hydrolyzed, and amide nitrogen determined. The dispersions in sodium salicylate were made up to one-fifth saturation with magnesium sulphate and all the precipitated material was washed, hydrolyzed and analyzed. Earlier work had shown that one-fifth saturation with magnesium sulphate precipitated the protein fraction that was highest in amide nitrogen content. The fraction not precipitated by this concentration, but precipitated by one-half saturation, was much lower in amide (12). The lower salt concentration was used, therefore, because it differentiated best between the fractions considered to belong to the true gluten complex and those considered as distinct.

It was necessary, however, to determine whether the protein that was considered as not belonging to the gluten complex was as distinct when isolated by solubility methods as it was when isolated by precipitation. For this reason, duplicate gluten balls were extracted with 1% sodium salicylate, which removes only the more soluble portion of the gluten protein, and the extract was concentrated and hydrolyzed. Amide nitrogen was determined on the hydrolysate.

The amide results for the two experiments, plotted against the midpoints of the gluten fractions, are presented in Fig. 3. Results obtained with a

similar flour by use of fractional precipitation methods (12) are also included in Fig. 3.

These results show that the distribution of amide nitrogen in successive gluten protein fractions is similar, whether the fractionation is carried out by solubility or precipitation methods. The latter method results in a sharper

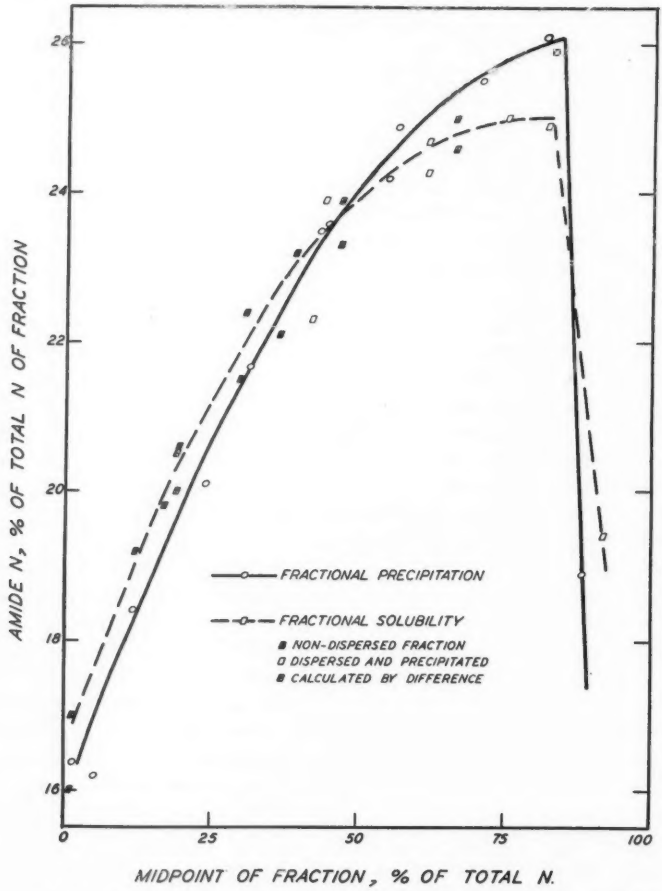

FIG. 3. *Amide nitrogen in gluten fractions obtained by fractional solubility and by fractional precipitation methods.*

separation of specific fractions, however, since there is no question of contamination of successive fractions by small amounts of unextracted portions of more soluble protein. This is shown by the fact that all but one of the points that were obtained by analysis of non-dispersed protein fall above the

general curve obtained by precipitation (Fig. 3), while all but one of those obtained by analysis of dispersed protein fall below it.

As a result of somewhat incomplete extraction, the non-dispersed protein contains small amounts of the more soluble fractions, which are, for the most part, higher in amide than the less soluble protein. This tends to raise the amide content of the non-dispersed fractions. Similarly, in precipitating the protein dispersed in salicylate, small amounts of the most soluble fractions, which are low in amide, are carried down with the precipitate. This tends to lower the amide content of the dispersed fractions, the effect being most marked with the smallest fractions. While each fraction obtained by total precipitation of the protein dispersed in various concentrations of salicylate shows this effect, only one fraction obtained by fractional precipitation can show it, because by the latter method the fractions are obtained successively from the same dispersion, and not separately from different dispersions. Thus the results obtained with fractional precipitation methods do not show a depression in amide content of the more soluble portions.

The results obtained with the concentrated 1% extract agree very well with those for the final precipitated fractions. Extraction was almost certainly incomplete with 1% salicylate, placing the midpoint of the fraction too far to the right on the graph. Had extraction been complete, the sharp breaks in the curves would have agreed even better than they do.

It has already been stated that the differences between the amide content of the various portions must be the result of differences in amide content of the fractions removed by progressively more concentrated solutions. The amide content of such extracts can be calculated from the weights of total and amide nitrogen. Furthermore, the calculations can be duplicated because the fraction in question appears once in the non-dispersed, and once in the dispersed, portion. The results for one experiment together with corresponding midpoints are presented in Table IV. These results have also been plotted in Fig. 3, and substantiate in every way the conclusions drawn from the direct analytical results.

TABLE IV

AMIDE CONTENT OF SPECIFIC FRACTIONS, OBTAINED BY CALCULATION OF DIFFERENCES IN SUCCESSIVE FRACTIONS

Concentrations of sodium salicylate between which solubility of fraction lies	Midpoint of fraction, % of total nitrogen	Amide nitrogen content	
		Non-dispersed	Dispersed
8–5	19.0	20.0	20.5
5–3	47.4	23.9	23.3
3–2	66.2	24.6	25.0

Discussion

It is now possible to consider the findings and conclusions of Blish as a result of fractional solubility studies. Two principal experiments are involved.

In the first experiment, Blish reports that more than 30% of the flour protein was extracted with water and that successive "fractions" of this extract did not vary in chemical composition. This was taken as definitely contradicting the evidence afforded by fractional precipitation data of the type presented by McCalla and Rose.

It was pointed out in the original paper that approximately 20 to 30% of the flour protein did not belong to the true gluten protein complex. Actually, 17.4% of the total nitrogen of one of the flours was "extracted" with water during gluten washing, and 16.1% more was extracted from the gluten with distilled water. Thus 33.5% of the flour nitrogen did not belong to the gluten complex. It is now apparent that the 16.1% extracted from this gluten was too low, as only a single extraction was made. That the successive "fractions" of the water-extracted protein did not vary in composition is to be expected, as these are not fractions, in the sense that we use the term, but are rather portions of the same fraction. In all our work it has been found that successive portions of the protein extracted with one solvent possess the same chemical composition. Thus we can agree with Blish's results, but not with his interpretation. Furthermore, his results were obtained with protein that did not belong to the true gluten complex, and therefore could not be accepted as definite contradiction of results or conclusions based on this complex, even if he had been dealing with distinct fractions.

In the second experiment (2), a single sample of flour was progressively extracted with sodium salicylate solutions of increasing concentration. A comparison of the chemical composition of the fractions revealed no evidence of progressive and systematic variations in chemical properties. The data were not presented, but it was concluded that they supported the idea that there are few components or component groups. As already described in a previous section, a similar experiment was carried out during the present studies. In this work single extractions of gluten with the lower concentrations of salicylate failed to remove more than small portions of the fractions specifically soluble in these concentrations, with the result that the remainder was carried over to successive extracts. The final result of this was a marked flattening of the amide curve, and a loss of individuality of fractions. Repeated extraction with a single concentration of salicylate, followed by repeated extraction with the next higher concentration, brought the results closer to those obtained by other methods, but in this case denaturation of the protein was so apparent that there was undoubtedly overlapping in the solubility of fractions that were originally distinct. We should hesitate to draw conclusions in support of the earlier hypothesis from these data, but we cannot accept the results as indicative of the true nature of gluten. On the basis of the results presented in the preceding section, the failure of these to conform to those previously presented can be reasonably explained; and the technical difficulties met in studying such fractions engenders little confidence in the results.

As a result of the present study it must be concluded that the technique involved in fractional solubility studies is much less satisfactory than that involved in precipitation. As successive extractions with low concentrations of salicylate not only increase the amounts of protein removed, but also denature the non-dispersed portions, complete extraction of any specific fraction is rendered difficult, and the complete removal of a second fraction is apparently impossible by our present methods. This is not so when dealing with relatively concentrated solutions, because penetration is rapid and complete. In this range, the results with individual fractions obtained by solubility methods agree with those obtained using precipitation methods.

As Blish points out, two interpretations of the results are possible when there is a systematic variation in physical and chemical properties of successive fractions. In the previous publication (12), these were interpreted as indicating an indefinite number of components in a single protein complex. Blish's viewpoint, however, is that there are relatively few main fractions, although the question as to the similarity of components within fractions is left open. The first view implies many distinct fractions, the second, mixtures containing progressively varying proportions of a few fractions. The present work on fractional solubility does not permit of final and dogmatic conclusions, but the results are in agreement with those obtained by fractional precipitation. The fractions could not have been the result of adsorption of one main component on another, as dispersion of a specific fraction only is effected. The possibility of the complex formation described by de Jong (6) influencing the formation of the fractions isolated cannot be dismissed; but preliminary results of an investigation designed to test this suggestion indicate that fractions occurring at any specific point in the gluten complex are of the same chemical nature, regardless of the pH of the salicylate solution. Further discussion of this phase of the work is unwarranted at the present time.

The possibility that the fractions might be formed as a result of overlapping solubilities of a few components implies that these main components must vary in solubility within themselves. If such variation is sufficient to produce fractions such as were isolated in the present study, we are probably justified in considering the different portions of the components as individual fractions.

It is perhaps well to repeat Blish's warning that a new discovery in this field may invalidate many of the conclusions reached as a result of previous studies. A final statement as to the composition of the gluten protein is impossible at the present time, but until more exact separation of the fractions is accomplished, we prefer to maintain the position outlined in the previous paper (12), and consider the main gluten protein as a single complex that can be divided into many fractions differing systematically in both physical and chemical properties.

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VARIETAL DIFFERENCES IN BARLEYS AND MALTS

IV. COMMONLY MEASURED PROPERTIES AND THEIR CORRELATIONS WITH NITROGEN AND 1000-KERNEL WEIGHT[1]

By W. O. S. Meredith[2] and J. Ansel Anderson[3]

Abstract

Samples representing 12 varieties of barley grown at 12 widely separated experimental stations in Canada were malted and subsequently analyzed. Varietal differences were found in steeping rate, malting loss, sprouts, extract, wort nitrogen and diastatic activity.

O.A.C. 21 and Mensury, varieties which Canadian maltsters prefer, gave high values for all six properties. Olli, which maltsters consider promising, gave still higher values. Pontiac equalled O.A.C. 21 only in diastatic activity, and the remaining six-rowed, rough-awned variety, Peatland, which has proved less satisfactory, gave lower values for all properties, and this inferiority was more apparent when adjustments were made for its high nitrogen content. In general, the six-rowed, smooth-awned varieties, particularly Regal and Wisconsin 38, gave much lower values. However, Nobarb was only 1% lower in extract than O.A.C. 21, and Velvet equalled the latter in wort nitrogen and diastatic activity. The two-rowed varieties, Charlottetown 80, Hannchen and Victory, were higher in extract but lower in other malt properties than O.A.C. 21. Hannchen, of which considerable quantities are malted in the United States, proved most similar to O.A.C. 21.

Amongst the *inter-varietal* correlations between malt properties and nitrogen content or 1000-kernel weight of the barley, only the partial correlations for diastatic activity and 1000-kernel weight, independent of nitrogen, proved significant ($r = 0.609$). Amongst the *inter-station* correlations, indicative of *intra-varietal* associations, those for nitrogen and extract ($r = -0.957$), nitrogen and diastatic activity ($r = 0.962$), and nitrogen and wort nitrogen ($r = 0.764$), surpassed the 1% level of significance; whereas those for nitrogen and steeping time ($r = -0.637$), nitrogen and malting loss ($r = 0.694$), and 1000-kernel weight and steeping time ($r = 0.652$), surpassed the 5% level. The inter-station multiple correlation coefficient for steeping time and nitrogen and 1000-kernel weight ($R = 0.840$) proved highly significant. The corresponding multiple correlation coefficient for extract was not significantly higher than the coefficient of correlation with nitrogen alone.

The investigations described in this series of papers were undertaken with the object of collecting an adequate body of data for the statistical examination of the correlations between various barley and malt properties, and of studying the bearing of these on the problem of evaluating the malting quality of new varieties of barley. For this purpose 144 samples of barley and the malts made from them are being analyzed. The samples represent 12 varieties of barley grown at each of 12 widely separated experimental stations in Canada.

The first three papers of this series dealt with the total nitrogen and nitrogen fractions of the barley (2), the saccharifying activities of the barley and malt (14), and the correlations between these properties (7). The present paper describes the methods used in making the malts, and deals mainly with the

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[2] *Senior Research Assistant, Associate Committee on Grain Research, Department of Plant Science, University of Manitoba, Winnipeg.*

[3] *Biochemist, Division of Biology and Agriculture, National Research Laboratories, Ottawa.*

rate of steeping, malting loss, extract, wort nitrogen and diastatic activity, and their correlations with total nitrogen content and 1000-kernel weight of the barley. These properties and a number of the relations have been studied by previous investigators (8–12, 14, and others), so that this paper presents few new generalizations. It is published mainly with the object of presenting data that provide additional information on the 12 varieties we are studying, and to which we shall also have occasion to refer in other papers of this series.

Materials and Methods

Materials

The barley samples used in the present investigation were described in detail in Part I of this series of papers (2). Briefly, they consist of 144 samples representing 12 varieties grown at each of 12 widely separated experimental stations in Canada. The varieties are listed in Table I and the stations in Table III.

Malting Method

In order to control systematic errors that might arise in malting a series of samples which required three months to complete, the malting was carried out in the following manner. Each batch of malt contained 12 samples representing the 12 varieties grown at one station. The samples were arranged in random order within batches and the batches were malted in random order. The first replicates were made in the first 12 batches and the second replicates in the succeeding 12 batches. It will be apparent that these methods provide for an unbiased comparison of the varieties.

Before malting, the thin barley and screenings were removed from each sample by passing it through a ring grader, with rings 5/64 in. apart, driven at constant speed by means of an electric motor and a reducing gear. The amount of plump barley recovered is reported as a percentage of the original sample weight.

The cleaned barley was then divided into two sub-samples by means of a Boerner sampler, and an aliquot part of each sub-sample, representing 25 gm. of barley dry matter, was used to determine the steeping time required to bring the moisture content of the barley to 46%. A larger aliquot part of each sub-sample, representing 250 gm. of barley dry matter, was subsequently malted in the laboratory equipment described by Anderson and Rowland (4).

The samples were steeped at 50° F. to a moisture content of 46%. They were then placed in the germination chamber, which was operated at an air temperature of 53° F. After 64 hr. each sample was watered to a moisture content of 48%, the amount of water required varying between 15 and 25 gm. After 144 hr. the samples were transferred to the kiln and dried under the following conditions: 0 to 6 hr., temperature rising at constant rate from 85° to 120° F.; 6 to 22 hr. at 120° F.; 22 to 30 hr., temperature rising at constant rate from 120° to 150° F.; 30 to 40 hr. at 150° F.; 40 to 42 hr., temperature rising at constant rate from 150° to 175° F.; 42 to 46 hr. at 175° F.

Malting loss and percentage sprouts (roots) were determined immediately after kilning. The sample was kneaded for one minute in a small bag. The roots that were rubbed off by this process were removed by sifting, and roots and finished malt were weighed. The percentage malting loss was calculated from data on the weights and moisture contents of the barley sample and the finished malt. The figures reported for sprouts are rather low, since some of these are rubbed off and lost during the kilning process.

The authors also wish to point out that the malts were somewhat undermodified. As a result it seems probable that maximum extract yields and complete development of enzymatic activity were not obtained. However, as previous investigations (3, and earlier papers in that series) have shown that the comparative order of varieties is little affected by moderate changes in malting conditions, we believe that the comparative results of the present investigation were not materially affected by the fact that the malts were not fully modified.

Analytical Methods

Determinations of the nitrogen content and 1000-kernel weight of the barley were made on cleaned samples. The former was determined by a Kjeldahl method on duplicate samples of barley ground separately. The latter was determined by weighing out duplicate 25-gm. samples and counting the number of kernels in each.

The following properties were determined by the Official Method of the American Society of Brewing Chemists (1): malt extract and moisture; color, odor, clarity, and speed of filtration of the wort; and saccharification time. Diastatic activity was determined by a ferricyanide modification of the Official Method (5). Wort nitrogen was determined on a 25-ml. aliquot of the laboratory wort by adding a few drops of acid, evaporating to a thin syrup, and making a Kjeldahl determination. It is reported as a percentage of the malt dry matter.

Results and Discussion

Varietal Differences

The results of the investigation are summarized in Table I as means, over all stations, for each variety. Owing to the differential effect of environment on varieties, these did not all fall in the same order with respect to any property at all stations. It was therefore necessary to resort to statistical analyses in order to determine whether the differences between varietal means could be considered significant. The results of the statistical analyses are given in a later section (Table VI), but are summarized in the last line of Table I as necessary differences between means required for a 5% level of significance, *i.e.*, for odds of 19 to 1 that a real difference between varieties is operating to spread the means.

A comparison of the necessary differences with the actual differences between the means for the individual varieties leaves no room for doubt that varietal differences exist with respect to each property measured. The results of the

TABLE I

BARLEY, MALTING, AND MALT PROPERTIES, MEANS FOR EACH VARIETY

Class	Variety	Barley			Malting data			Malt		
		Total nitrogen, %	1000-kernel wt., gm.	Plump barley, %	Steeping time, hr.	Malting loss, %	Sprouts, %	Extract, %	Wort nitrogen, %	Diastatic power, °L.
Six-rowed, rough-awned	O.A.C. 21	2.242	31.0	74.3	75	7.4	2.2	73.9	0.82	127
	Mensury, Ott. 60	2.303	32.1	77.1	78	7.3	2.1	73.9	0.85	129
	Olli	2.215	29.6	63.5	55	7.7	2.1	76.0	0.93	153
	Peatland	2.471	28.6	66.7	80	7.1	1.8	73.2	0.80	120
	Pontiac	2.278	32.5	79.3	78	6.9	1.9	72.5	0.76	131
Six-rowed, smooth-awned	Nobarb	2.194	33.8	80.2	82	6.4	1.6	72.9	0.66	100
	Regal	2.352	31.6	76.7	86	7.0	1.7	71.1	0.74	85
	Velvet	2.372	31.1	76.8	80	6.6	1.7	72.3	0.80	124
	Wisconsin 38	2.240	32.4	86.1	91	6.6	1.6	71.2	0.64	95
Two-rowed, rough-awned	Charlottetown 30	2.298	35.1	82.5	78	7.6	2.1	76.1	0.76	100
	Hannchen	2.215	35.6	83.9	79	7.2	1.9	76.9	0.80	116
	Victory	2.228	36.1	83.9	78	7.1	2.1	75.9	0.71	103
Mean over all varieties		2.284	32.4	77.0	78	7.1	1.9	73.8	0.77	115
Necessary difference, 5% level		0.079	1.5	8.5	5	0.4	0.3	0.7	0.04	11

investigation are thus in agreement with those of other investigators who have studied these properties.

Canadian maltsters have found by experience that O.A.C. 21 and Mensury Ott. 60 are very satisfactory for the production of brewers' malt for domestic consumption. These varieties grow readily during the malting process, as is shown by data for malting loss and sprouts, and produce malts characterized by fairly high extract yields, high diastatic activity, and high percentages of wort nitrogen.

As a result of many laboratory tests and some commercial cage malting tests, the maltsters are inclined to consider Olli a very promising variety, and commercial scale tests will be made with it in the near future. The variety is a comparatively new introduction which matures very early and is suited only to northern districts. It gives an extremely high extract yield for a six-rowed variety and is characterized by high enzymatic activity as is shown by the data for diastatic power, wort nitrogen, malting loss and sprouts. It tends to produce grain of low nitrogen content but has rather small kernels.

Peatland has been malted commercially and was at one time considered a promising variety. It is now considered of doubtful value for malting purposes, largely, we believe, because of its tendency to produce grain of very high nitrogen content. In other respects it appears to have much the same properties as O.A.C. 21.

Information on the malting quality of Pontiac is confined to the results of laboratory tests. The variety does not seem promising since it grows slowly and the malt is rather low in extract yield and percentage of wort nitrogen.

Canadian maltsters do not consider any of the four smooth-awned varieties listed suitable for malting purposes. On the other hand large quantities of Velvet and Wisconsin 38 are malted in the United States, and of these two varieties the former is considered superior (11). Velvet appears to grow rather more slowly than O.A.C. 21. It also yields lower extract values, but is equal to O.A.C. 21 with respect to diastatic activity and wort nitrogen. Nobarb gives a higher extract than Velvet, but it and the other two smooth-awned varieties give lower values for the other main malt properties.

The two-rowed varieties are characterized by high 1000-kernel weight and high extract yield. Since the amounts of two-rowed barley malted in Canada are extremely small, information on the comparative malting qualities of these three varieties is confined largely to the results of the present investigation. It is interesting to note, however, that Hannchen, of which considerable quantities are malted in the United States, is higher in diastatic activity and percentage wort nitrogen than the other two varieties.

No varietal differences were demonstrated with respect to certain minor properties of the malt and wort. In these circumstances it seems necessary only to list the properties in question, together with average values for them: malt moisture, 3.3%; color of wort, 1.6 Lovibond units; odor of wort, aromatic; appearance of wort, clear; speed of filtration, normal; and time of conversion, less than 5 min.

Varietal Differences at Equal Nitrogen Contents

The interpretation of the data on malt properties is complicated by the existence of varietal differences in total nitrogen content and of intra-varietal relations between nitrogen and the malt properties. In these circumstances it seems useful to compare the varieties at equal nitrogen contents. This can be effected by determining the coefficients of regression of each malt property on total nitrogen and using these to adjust the varietal means for each property to the values corresponding to a total nitrogen content of 2.28%, which figure represents the mean nitrogen content of all varieties.

It is apparent that this procedure will be useful only if the regression coefficients for the individual varieties are almost identical, since if they are not, the comparative order or relative positions of the varieties would depend upon the nitrogen content at which the comparison of adjusted means was made. Statistical analyses, reported in a later section (Table VII), showed that the varietal regression coefficients for extract on total nitrogen did not differ significantly, nor did those for wort nitrogen on total nitrogen. The adjusted means for these properties were accordingly calculated and are reported in Table II. For the information of those who consider an index of nitrogen modification useful, wort nitrogen is also reported as a percentage of total nitrogen of the barley. In this connection it should be noted that total wort nitrogen rather than the more commonly determined permanently soluble nitrogen was used in calculating the indices of nitrogen modification.

TABLE II

VARIETAL MEANS FOR MALT PROPERTIES ADJUSTED BY CALCULATION TO VALUES CORRESPONDING TO TOTAL NITROGEN CONTENT OF BARLEY OF 2.28%

Class	Variety	Extract, %	Wort nitrogen		Diastatic activity, °L.	
			Per cent of dry matter	Per cent of total nitrogen	At total nitrogen of 2.28%	At total nitrogen of 1.80%
Six-rowed, rough-awned	O.A.C. 21	73.7	0.83	36.4	128	93
	Mensury, Ott. 60	74.0	0.84	36.8	128	90
	Olli	75.7	0.94	41.2	158	122
	Peatland	74.1	0.76	33.3	105	66
	Pontiac	72.5	0.76	33.3	131	92
Six-rowed, smooth-awned	Nobarb	72.5	0.68	29.8	105	79
	Regal	71.5	0.73	32.0	82	60
	Velvet	72.7	0.77	33.8	116	75
	Wisconsin 38	71.0	0.65	28.5	97	78
Two-rowed, rough-awned	Charlottetown 80	76.0	0.75	32.9	100	87
	Hannchen	76.6	0.81	35.5	121	88
	Victory	75.7	0.72	31.6	105	86
Mean, over all varieties		73.8	0.77	33.8	115	85
Necessary difference, 5% level		0.5	0.03	1.3	11	8

Statistical analyses showed that the varietal regression coefficients for diastatic activity on nitrogen content differed significantly. (This point is illustrated by the scatter diagrams given in Fig. 2 of Part III of this series.) Accordingly, adjustment of the mean diastatic activities to a given nitrogen content presents an incomplete picture of varietal differences. In order to overcome this difficulty, adjusted means were calculated not only for a nitrogen content of 2.28% but also for 1.80%. These values probably represent about the lower and upper limits of the nitrogen content of barley malted commercially in Canada. The two sets of adjusted varietal means for diastatic activity are given in the last two columns of Table II.

The effect of adjusting for differences in total nitrogen content is most marked with respect to Peatland. After adjustment this variety has as high an extract yield as O.A.C. 21 but gives lower values for both wort nitrogen and diastatic activity. It thus appears that when comparisons are made at equal nitrogen contents, Peatland is low in enzymatic activity, which may well be an additional reason for its failure to find favor with the maltsters.

The data for total wort nitrogen as percentage of total barley nitrogen form a so-called index of modification. Since all varieties were malted under the same conditions it is apparent that O.A.C. 21, Mensury, Hannchen, and particularly Olli, modify much more readily than the other varieties, and these are the four varieties that seem most satisfactory to the maltsters. It may also be argued that the lower values given by other varieties indicate that these were not malted advantageously, and that their indices of modification could be raised by changing the malting conditions. Previous investigations (3, and earlier papers in that series) suggest that there is some truth in this hypothesis. We believe, however, that varietal differences in indices of modification would persist even if each variety were malted under optimum conditions. This is the opinion held by Thunaeus and Schröderheim (15) as a result of their investigations of Swedish barleys.

The data for diastatic activity show that, owing to differences in the varietal regression coefficients, the comparative orders of the varieties are not identical when adjustments are made to correspond with different nitrogen levels. It will be noted, however, that at both levels Olli gives by far the highest values, and is followed by a group of four varieties, O.A.C. 21, Mensury, Pontiac and Hannchen. At both nitrogen levels Regal also yields the lowest values for diastatic activity.

Station Differences

The results of the investigation are summarized in Table III as means, over all varieties, for each station. The stations are listed in order of increasing nitrogen content of the barley in order to facilitate examination of the relations between nitrogen and other properties, a subject that is discussed in the next section.

The data show that environmental differences among the stations resulted in the production of a series of samples covering a wide range of values with

TABLE III

BARLEY, MALTING, AND MALT PROPERTIES, MEANS FOR EACH STATION

Station	Barley			Malting data			Malt		
	Total nitrogen, %	1000-kernel weight, gm.	Plump barley, %	Steeping time, hr.	Malting loss, %	Sprouts, %	Extract, %	Wort nitrogen, %	Diastatic power, °L.
Nappan	1.540	34.8	92.2	99	6.8	1.6	78.0	0.61	63
Fredericton	1.738	34.8	84.7	83	6.5	1.7	76.3	0.65	85
Ste. Anne de Bellevue	1.932	32.9	79.3	74	7.0	1.6	75.5	0.78	100
Ste. Anne de la Pocatière	2.278	33.5	89.5	77	6.9	2.0	74.3	0.82	121
Lethbridge	2.294	33.5	86.6	84	7.2	2.0	74.7	0.73	116
Winnipeg	2.333	30.6	65.0	75	7.2	2.3	73.0	0.75	105
Brandon	2.357	28.0	84.0	77	7.0	2.0	73.9	0.78	117
Guelph	2.381	32.5	50.3	65	6.6	1.8	72.8	0.87	122
Ottawa	2.526	28.0	69.7	81	7.4	1.9	73.3	0.87	133
Lacombe	2.668	32.5	82.9	75	7.1	2.0	71.9	0.79	140
Beaver Lodge	2.674	31.8	78.0	74	8.0	2.4	70.5	0.78	149
Gilbert Plains	2.687	30.8	67.3	76	7.4	1.7	71.6	0.81	133
Mean over all stations	2.284	32.4	77.0	78	7.1	1.9	73.8	0.77	115
Necessary difference, 5% level	0.079	1.5	1.5	5	0.4	0.3	0.7	0.04	11

respect to each of the properties studied. The spread in malting quality is wider than that between the best and poorest samples malted commercially in Canada. However, the stations were selected with the object of obtaining wide differences in malting quality, in order to facilitate the demonstration of such relations between the various barley and malt properties as may exist.

Correlations between Total Nitrogen and Other Properties

The correlations between total nitrogen and other properties were examined by calculating the correlation coefficients for varietal means and station means. The resulting statistics are given in Table IV. None of the coefficients for varietal means attained the 5% level of significance, so that the investigation yields no evidence of inter-varietal relations between nitrogen and other properties.

TABLE IV

INTER-VARIETAL AND INTER-STATION CORRELATIONS FOR NITROGEN CONTENT AND OTHER PROPERTIES

Property	Correlation coefficient	
	Inter-varietal	Inter-station
1000-kernel weight	−.552	−.519
Plump barley	−.338	−.440
Steeping time	.238	−.637*
Malting loss	−.041	.694*
Sprouts	−.061	.640*
Extract	−.380	−.957**
Diastatic activity	−.039	.962**
Wort nitrogen as % of dry matter	.166	.764**

NOTE: *In this and later tables ** denotes that the 1% level, and * that the 5% level of significance is attained.*

Reference to Table III, which gives the mean values for each station, will show that as the total nitrogen increases, malt extract tends to decrease, and malting loss, sprouts, wort nitrogen and diastatic activity tend to increase. The degree of association between nitrogen and these other properties is summarized by the correlation coefficients between station means given in the last column of Table IV. It is quite close for extract and diastatic activity, but very considerably lower for the other properties. All the associations, except that between nitrogen and steeping time, have been discussed by earlier investigators and further comment on them seems unnecessary.

Correlations between 1000-kernel Weight and Other Properties

The inter-varietal and inter-station coefficients of correlation between 1000-kernel weight and other properties are given in the first two columns of data in Table V. Owing to the association between 1000-kernel weight and nitrogen it appeared that these statistics might be misleading, since they might merely, or at least partially, reflect correlations with nitrogen, rather than represent actual correlations with 1000-kernel weight. The possible effects of nitrogen were therefore eliminated by calculating the partial correlation coefficients for 1000-kernel weight and each property, independent of nitrogen. These are listed in the last two columns of Table V.

A comparison of the simple and partial coefficients shows that in some instances the former did partially reflect correlations with nitrogen, whereas

in other instances the association with nitrogen tended to mask the true association with 1000-kernel weight. Only the partial correlation coefficients appear to merit further discussion.

TABLE V

INTER-VARIETAL AND INTER-STATION CORRELATIONS FOR 1000-KERNEL WEIGHT AND OTHER PROPERTIES, AND PARTIAL CORRELATIONS INDEPENDENT OF NITROGEN CONTENT

Property	Correlation coefficient		Partial correlation coefficient	
	Inter-varietal	Inter-station	Inter-varietal	Inter-station
Total nitrogen	−.552	−.519	—	—
Plump barley	.361	.933**	.191	.840**
Steeping time	.289	.756**	.525	.652*
Malting loss	−.099	−.173	−.146	.304
Sprouts	.120	−.200	.104	.202
Extract	.439	.600	.297	.416
Diastatic activity	−.486	−.434	−.609*	.276
Wort nitrogen as % of dry matter	−.490	−.654*	−.485	−.466

Amongst the inter-varietal partial correlations only that for diastatic activity is significant, but the association between the two variables is by no means close. We may assume, however, that there is some tendency for varieties that are lower in 1000-kernel weight to be higher in diastatic activity.

The inter-station partial correlation coefficients show that there is a fairly close intra-varietal relation between percentage plump barley and 1000-kernel weight of the plump barley. This was to be expected and it seems peculiar that no such inter-varietal relation exists.

It is also apparent that there is an inter-station association between 1000-kernel weight and steeping time, which indicates that within any variety samples of smaller 1000-kernel weight tend to absorb water more rapidly. Although the corresponding inter-varietal correlation coefficient does not attain the 5% level of significance, it is not far below this, and thus suggests that a similar inter-varietal association may well exist. On common sense grounds we should expect both an inter- and an intra-varietal relation between 1000-kernel weight, which is a measure of kernel size, and rate of water absorption. The surprising thing is that the degree of association between these two variables is not greater. It is thus apparent that although 1000-kernel weight is one of the factors controlling rate of water absorption, there must be other factors. Data presented in the next section show that some of these factors are associated with the nitrogen content of the grain.

Multiple Correlations between Certain Properties, Nitrogen and 1000-kernel Weight.

Steeping time. The simultaneous association between steeping time, nitrogen and 1000-kernel weight was investigated by calculating multiple correla-

tion coefficients representing the correlation between steeping time as determined and steeping time as predicted from nitrogen and 1000-kernel weight. The data were also analyzed in order to determine whether the prediction based on two independent variables could be considered significantly better than prediction based on one alone (see Table IX). This proved true for the inter-station multiple coefficient ($R = 0.826$), which surpassed the 1% level of significance. It thus appears that within each variety the time required for the absorption of 46% of moisture depends not only on the kernel weight but also on some other factor, or factors, associated with nitrogen content. However, since the multiple correlation coefficient is not very high it also follows that some factor, or factors, associated with neither nitrogen nor 1000-kernel weight, also play a part in controlling rate of water absorption.

The inter-varietal multiple correlation coefficient ($R = 0.546$) fails to attain the 5% level of significance. Moreover, it is little higher than the partial correlation coefficient for steeping time and 1000-kernel weight, independent of nitrogen ($r = 0.525$). It is thus apparent that between varieties, factors closely associated with nitrogen content have little influence on steeping time. It appears, however, that 1000-kernel weight has some influence although it is not a factor of major importance.

Extract. As Bishop and Day (10, and earlier papers) have made use of both nitrogen and 1000-kernel weight for predicting extract, it seemed advisable to determine whether our data would show a significant increase in the precision of prediction through the introduction of 1000-kernel weight as a second independent variable. The inter-station multiple correlation coefficient ($R = 0.964$) proved to be slightly, but not significantly, higher (see Table IX) than the coefficient of correlation between extract and nitrogen alone ($r = -0.957$). It thus appears that the advantage to be gained by the introduction of the second independent variable is very small.

The inter-varietal multiple correlation coefficient failed to attain, or even to approach closely, the 5% level. There are thus no grounds for believing that varieties that are low in nitrogen and high in 1000-kernel weight will tend to be higher in extract yield than varieties having the opposite characteristics.

Diastatic activity. The simultaneous association between diastatic activity, nitrogen, and 1000-kernel weight, was discussed in Part III (7).

Other Relations

The data presented in this and preceding papers (2, 7, 14) make possible the investigation of a number of other correlations between barley and malt properties. A study of these is being undertaken and the results will be presented in further papers of this series.

Statistical Analyses

The methods used for analyzing the data reported in this paper are the same as those used under similar circumstances for analyzing data given in Parts I and III. In these circumstances it seems unnecessary to discuss the statistics in detail, although it seems advisable to present them in order to substantiate statements made previously in this paper.

The variance of the data for each property was analyzed separately and the resulting mean squares are given in Table VI. Before varietal means were adjusted for differences in total nitrogen (see Table II), the homogeneity of varietal regression coefficients was tested by means of analyses of residual inter-station, intra-varietal variance. The results of these tests are given in Table VII. Only the varietal regressions for diastatic activity on nitrogen proved unhomogeneous. Varietal means for extract and wort nitrogen were adjusted for differences in total nitrogen and the significance of the differences between the adjusted means was tested in the usual way by calculating the adjusted residual mean squares. These are given in Table VIII. Analyses of var-

TABLE VI

ANALYSES OF VARIANCE FOR BARLEY, MALTING AND MALT PROPERTIES: MEAN SQUARES

Property	Variance due to		
	Stations	Varieties	Interaction
Total nitrogen, %	1.651**	0.079**	0.009
1000-kernel weight, gm.	44.71**	65.97**	3.55
Plump barley, %	1769**	549**	108
Steeping time, hr.	801.2**	905.6**	41.9
Malting loss, %	1.775**	1.977**	0.265
Sprouts, %	0.789**	0.608**	0.095
Extract, %	53.57**	46.56**	0.60
Diastatic power, °L.	7052**	4372**	166
Wort nitrogen as % of dry matter	0.0725**	0.0770**	0.0018
Degrees of freedom	11	11	121

TABLE VII

TEST OF HOMOGENEITY OF VARIETAL REGRESSION COEFFICIENTS BY ANALYSIS OF RESIDUAL VARIANCE

Variance due to	Degrees of freedom	Mean squares		
		Extract	Wort nitrogen	Diastatic activity
Differences among varietal regression coefficients	11	0.333	0.0025	686.5**
Deviations from individual varietal regressions	120	0.808	0.0039	119.0

TABLE VIII

TEST OF SIGNIFICANCE OF VARIANCE IN EXTRACT AND WORT NITROGEN ADJUSTED FOR DIFFERENCES IN TOTAL NITROGEN

Variance due to	Degrees of freedom	Residual mean squares	
		Extract	Wort nitrogen
Varieties	11	40.816**	0.07515**
Stations	11	4.630**	0.03078**
Remainder	120	0.415	0.00131

iance were also used to determine whether the prediction of steeping time, extract, and diastatic activity, from barley nitrogen could be improved by the introduction of 1000-kernel weight as a second independent variable. The resulting statistics are given in Table IX.

TABLE IX

TEST OF SIGNIFICANCE OF DIFFERENCE BETWEEN SIMPLE COEFFICIENT OF CORRELATION WITH NITROGEN AND MULTIPLE COEFFICIENT OF CORRELATION WITH NITROGEN AND 1000-KERNEL WT.

Dependent variable	Variance accounted for by	Degrees of freedom	Mean square	
			Varieties	Stations
Steeping time	Total nitrogen	1	564	3576**
	Added effect of 1000-kernel wt.	1	2406	2169*
	Residual	9	777	341
Extract	Total nitrogen	1	74.14	539.29**
	Added effect of 1000-kernel wt.	1	38.76	8.66
	Residual	9	44.36	4.59
Diastatic activity	Total nitrogen	1	73	71714**
	Added effect of 1000-kernel wt.	1	17831*	445
	Residual	9	3355	602

Acknowledgment

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EFFECT OF PHYTOHORMONE DUSTS ON GROWTH AND YIELD OF WINTER WHEAT VARIETIES[1]

By G. P. McRostie[2], J. W. Hopkins[3] and N. H. Grace[4]

Abstract

Various concentrations of indolyl- and naphthylacetic acids were applied to ten varieties of winter wheat prior to planting in a replicated field trial, one-half of each plot receiving seed dusted with Ceresan plus phytohormone, and the other half receiving an equal quantity of seed of the same variety dusted with Ceresan only.

Some differences between the hormone-treated and untreated sub-plots in respect of early growth and subsequent density of stand were apparent to visual inspection. There were also statistically significant differences in respect of straw production, and of yield, weight per bushel, and nitrogen content of grain. The effects on grain yield were complicated by the differential response of varieties to the same treatment, and at the higher concentrations some depressions of yield resulted. On the average both chemicals tended slightly to reduce the nitrogen content of the grain produced.

The results as a whole seem to demonstrate the physiological activity of both substances tested when applied in this way. Further investigation of dosages, varietal characteristics, and seasonal effects, however, will be required before general conclusions can be reached.

Preliminary tests, by one of the authors, of the physiological activity of certain phytohormone chemicals when applied in the form of dusts to seeds prior to planting, have already been reported (1). In view of the promising indications obtained, it was desired to ascertain whether these substances had any appreciable effect on the growth and yield of crop plants under field conditions.

As winter wheat might perhaps be expected to respond particularly advantageously to any induced stimulation of germinative or early vegetative vigor, a replicated varietal trial of this crop, with and without phytohormone dust treatment, was laid down at the Ontario Agricultural College, Guelph, in the autumn of 1937. The present communication describes the results secured from this trial in the season 1937–38.

Experimental

The hormone trial was superimposed upon the annual field test of standard winter wheat varieties regularly conducted at the college. This was effected by planting one-half (selected at random) of each plot with seed dusted with Ceresan plus phytohormone, and the other half with an equal quantity of seed of the same variety dusted with Ceresan (an organic mercury fungicide) only. Ten varieties were included in the test, each replicated six times, the field arrangement being in the form of six randomized blocks. The whole experiment thus comprised 60 main plots and 120 sub-plots. Main plots

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Contribution from the Department of Field Husbandry, Ontario Agricultural College, Guelph, and the Division of Biology and Agriculture, National Research Laboratories, Ottawa.

[2] *Professor of Field Husbandry, Ontario Agricultural College, Guelph.*
[3] *Statistician, National Research Laboratories, Ottawa.*
[4] *Chemist, National Research Laboratories, Ottawa.*

were 18½ ft. long and ten rows wide, the rows being spaced 6 in. apart. Sub-plots were 18½ ft. long and five rows wide. The soil on which the test plots were located was of the Miami loam group formed on a coarse glacial drift, and characterized by good natural drainage. The surface soil was neutral in reaction, rather low in available phosphate and from medium to low in potash. It was in excellent tilth, the result of plowing down a heavy stand of alfalfa earlier in the season.

It was desired to test ten different hormone preparations, so that the number of treatments was the same as the number of varieties. However, in order to provide some information respecting possible differential responses of varieties to treatment, each phytohormone dust was applied to three replicate plots of each of two varieties. The six randomized blocks were thus of two types, which may be designated *A* and *B*. Representing the ten winter wheat varieties, which will be found listed by name in Table II, by the Roman numerals I to X, and the ten hormone preparations by the Arabic numerals 1 to 10, then in the three blocks of type *A*, the phytohormones were applied to the wheats as follows:

I.1, II.2, ... IX.9, X.10,

whereas in the three blocks of type *B* the combinations were:

I.6, II.7, ... VI. 1,. . . . . X.5.

Ceresan was used throughout at the rate of ½ oz. per bushel, whilst the phytohormone dosages were 2, 5 and 10 p.p.m. of indolylacetic, 2, 5, 10, 20, 50 and 100 p.p.m. of naphthylacetic, and 2 p.p.m. of a mixture of indolyl- and naphthylacetic acids. In each case the hormone concentration is expressed as parts per million by weight of seed treated. The dusts were applied a few hours before planting, each lot of seed and dust being thoroughly shaken together in a wide-mouthed glass bottle in order to bring about as uniform a distribution of the dust as possible.

Seeding took place on September 2, 1937, at the rate of two bushels per acre, and harvesting on July 18, 1938. At harvest time, one foot at each end and the two outside rows of each sub-plot were discarded in order to minimize border or competitive effects.

Results

Field notes on germination and early growth were taken ten days after sowing, and although these were in some instances rather conflicting, on the whole there seems to have been a stimulation of vegetative growth by all four concentrations of indolylacetic acid, *viz.*, 2, 5 and 10 p.p.m., and 2 p.p.m. plus 2 p.p.m. naphthylacetic, and also by 2 and 5 p.p.m. of naphthylacetic; whereas the higher concentrations of naphthylacetic, namely 10, 20, 50 and 100 p.p.m., apparently retarded early development. Similar conclusions are to be deduced from visual estimates of the relative stand on the individual sub-plots subsequent to the resumption of growth in the spring, recorded on

May 6, 1938. On the other hand, the final height attained by the crop was but little affected, the only noticeable differences being an average increase of 2.3 in. in the sub-plots of Dawson's Golden Chaff receiving 5 p.p.m. of indolylacetic acid, and a decrease of 2.0 in. in those of Red Rock receiving 100 p.p.m. of naphthylacetic. In neither case was there any corresponding effect on the second variety receiving the same treatment.

There was no actual lodging in any of the sub-plots, but a certain amount of leaning was in evidence in some varieties which, however, never affected more than 20% of the crop. This tendency was slightly accentuated in the sub-plots receiving the lower concentrations (2 and 5 p.p.m.) of indolylacetic acid.

After harvesting, the separate weights of straw and grain, and the weight per measured bushel of the grain from the individual sub-plots were recorded, after which quarter-pound samples of the grain from each sub-plot were forwarded to the National Research Laboratories, Ottawa, for determination of nitrogen content by the Kjeldahl method. The data thus secured were subjected to analyses of variance, with the results indicated in Table I. Since the experiment was of the split-plot type, the quantities thus analyzed were not the actual yields, nitrogen contents, etc., but the differences in these respects between the hormone-treated and untreated halves of each main plot, hereafter on occasion designated "responses". It should be mentioned therefore that the season proved to be a favorable one, and that the outturn of crop was good, the average yield of grain for the experiment as a whole being 48.5 bu. per acre, the average yield of straw 6866 lb. per acre, the average weight per bushel of the grain, 61.2 lb., and its average nitrogen content 2.20% (dry basis).

It will be observed from Table I that there were significant differences between the hormone-treated and untreated sub-plots in respect of all four

TABLE I

ANALYSIS OF VARIANCE OF RESPONSE OF WINTER WHEAT VARIETIES TO PHYTOHORMONE DUSTS

Source of variance	Degrees of freedom	Mean square			
		Straw production (000 omitted)	Grain yield	Weight per bushel	Nitrogen content
Between blocks:					
Between block types (1 d.f. interaction confounded)	1	5097	182.25*	0.87	.0216
Within block types (error (1))	4	2604	20.91	0.69	.0282
Within blocks:					
Average response	1	95600**	93.08*	3.95**	.0482*
Treatment differences	9	10923*	45.44*	0.82	.0234*
Interaction variety × treatment	9	3255	33.80*	0.69	.0075
Residual error (error (2))	36	3823	15.63	0.40	.0092

* *Exceeds mean square error, 5% level of significance.*
** *Exceeds mean square error, 1% level of significance.*

TABLE II

RESPONSE OF WINTER WHEAT VARIETIES TO PHYTOHORMONE DUSTS

Phytohormone treatment	Variety to which applied		Straw production, lb./acre	Grain yield, bu./acre			Wt. per bushel, lb.	Nitrogen content (dry basis), %
	Blocks A	Blocks B		Blocks A	Blocks B	Average		
2 p.p.m. indolylacetic	D.G.C., O.A.C. 61	O.A.C. 104	+1174*	+3.88	+5.11*	+4.50	+0.7*	−.01
5 p.p.m. indolylacetic	Dawson's Golden Chaff	Dawtas	+ 702*	+1.76	+1.22	+1.49	+1.0*	−.06
10 p.p.m. indolylacetic	D.G.C., O-24-12	Imperial Amber	+ 722*	+6.80*	+0.32	+3.56	+0.4	−.04
2 p.p.m. naphthylacetic	Dawbul	Red Rock	+ 554*	+3.15	+1.73	+2.44	+0.2	−.10*
5 p.p.m. naphthylacetic	Junior No. 6	Kharkov	+ 358	+1.05	+2.28	+1.66	−0.2	−.04
10 p.p.m. naphthylacetic	O.A.C. 104	D.G.C., O.A.C. 61	+ 187	+2.84	−5.21*	−2.38	+0.2	+.05
20 p.p.m. naphthylacetic	Dawtas	Dawson's Golden Chaff	+ 424	+5.39*	−3.07	+1.16	0.0	+.08*
50 p.p.m. naphthylacetic	Imperial Amber	D.G.C., O-24-12	− 164	−4.62*	−5.06*	−4.84*	−0.2	−.12*
100 p.p.m. naphthylacetic	Red Rock	Dawbul	− 267	+5.90*	−5.95*	−0.02	+0.2	+.02
2 p.p.m. ind. + naphthylacetic	Kharkov	Junior No. 6	+ 302	+3.74	+3.65	+3.69	+0.1	−.05
Necessary for 5% level of significance			505	4.56	4.56	4.74	0.7	.08
Average			+ 399*	+2.99*	−0.49	+1.25*	+0.26*	−.028*

* *Individually significant, 5% level.*

characters dealt with. The results would thus seem to demonstrate the undoubted physiological activity of the chemicals tested when applied in this way, as well as the existence of some differences in response to the individual treatments. They do not, however, permit of any sharp discrimination between chemicals or dosages, as will be apparent from the necessary differences given in Table II. To do this would require a considerably higher degree of replication, since the intrinsic accuracy of this particular trial was about the average for field experiments of this type, the standard error of a single sub-plot in respect of grain yield, for example, being 2.8 bu. per acre, or 5.8% of the mean. In the case of grain yield, moreover, the situation is further complicated by the significant interaction, *i.e.*, differential response of varieties to the same treatment, indicated in Table I.

Table II lists the varieties to which each phytohormone preparation was applied in the blocks of type *A* and *B*, and also shows the effect of each, as estimated from the differences between adjacent treated and control sub-plots, on the yield of straw and on the yield, weight per bushel, and nitrogen content of grain. In the case of grain yield, which was shown in Table I to be differentially affected by the same treatment when applied to different varieties, the results for each variety (average of three main plots) are included separately. As, however, the effects on the other three quantities did not exhibit any significant differences between varieties, the mean for both varieties (average of six main plots) is given in these cases.

Significantly increased straw production is to be noted in the sub-plots receiving all three concentrations of indolylacetic acid alone, the most pronounced effect being secured from 2 p.p.m. In this respect, indolyl- proved to be more effective than naphthylacetic acid, from which only one significant response was secured, at a dosage of 20 p.p.m. In no instance, however, was the final yield of straw significantly depressed by treatment with either substance. It would seem that, in the absence of any pronounced differences in height, increases in straw production must be attributed to a denser stand. This might result either from an improvement in germination or survival, or from additional tillering. It has already been noted that some such differences in stand were apparent to visual inspection.

The effects on grain yield are not as clear-cut, owing to the differential response of varieties to the same treatment. Such differentials are perhaps not altogether surprising, in view of similar effects noted in fertilizer trials, but it is believed that this is the first occasion on which they have been demonstrated in an experiment with phytohormones on varieties of the same plant species. In one instance this resulted in a complete reversal, 100 p.p.m. of naphthylacetic acid increasing the yield of the variety Red Rock by 5.90 bu. per acre, but simultaneously depressing the yield of Dawbul by 5.95 bu.

Although less effective than indolylacetic in modifying straw production, naphthylacetic acid had more influence on grain yield, six significant differences being noted in individual varieties. Four of these, however, were depressive at the concentrations used (10, 50 and 100 p.p.m.). Significant increases were also obtained from 2 and 10 p.p.m. of indolylacetic acid, but

in both cases this response was forthcoming from only one of the two varieties thus treated.

It is of interest to note that kernel size, as indicated by the weight per measured bushel, was also apparently slightly affected. Over the entire experiment, there is a small but significant difference of 0.26 lb. per bushel in favor of the hormone-treated sub-plots. However, this seems to be almost entirely attributable to the indolylacetic acid treatments, as the differences for naphthylacetic fail to attain significance either individually or collectively. The effect is in no case pronounced, the maximum increase of 1.0 lb. (5 p.p.m. indolylacetic) being only about 2% in excess of the corresponding control.

On the whole, the effect of the phytohormones has been slightly to depress nitrogen content, although the average difference, 0.028%, is only 1.3% of the mean of the control sub-plots (2.21%). The maximum difference, −0.12% at 50 p.p.m., is of the order of 5% of the control. The grain from all three indolylacetic acid treatments is lower in nitrogen content than that of the controls, but none of these differences is statistically significant. Both increases and decreases in nitrogen content are in evidence in the naphthylacetic acid plots, although the positive effects are associated with phytohormone concentrations that probably would not be employed in normal practice, and that may well have affected the absorptive capacity of the root system. The average result is in accordance with expectation, and may perhaps even be regarded as providing independent evidence of real effects on yield, since if nitrification proceeded at substantially the same rate in the treated and untreated sub-plots, the nitrogen absorbed by the higher-yielding plants on the former would have to be distributed over more straw and grain. On the other hand, even after the exclusion of the higher dosages, there seems to be no consistent relation between the differences in nitrogen content and those in grain yield. As was pointed out previously, however, comparisons between treatments are subject to relatively large experimental errors. Furthermore, the recorded yields may be affected by accidents of harvesting, handling, etc., of which the nitrogen content would be largely independent.

As was suggested earlier in this section, the foregoing results, taken collectively, would seem to demonstrate the undoubted physiological activity of the phytohormone dusts used, under the conditions of this experiment. At the same time, it is clear that further experimentation with respect to both dosage and varietal characteristics, as well as possible seasonal effects, will be necessary before general conclusions can be reached. In the meantime, however, it may be of interest to observe that the maximum apparent stimulation of grain yield of an individual variety, namely 6.80 bu. (10 p.p.m. indolylacetic acid on Dawson's Golden Chaff 0–24–12), is an increase of approximately 13% over the control, and that the maximum observed increase in straw production, 1262 lb. (2 p.p.m. indolylacetic on Dawson's Golden Chaff, O.A.C. 61), represents an increment of roughly 20%.

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HYBRIDIZATION OF *TRITICUM* AND *AGROPYRON*

V. DOUBLING THE CHROMOSOME NUMBER IN *T. VULGARE* AND F_1 OF *T. VULGARE* × *A. GLAUCUM* BY TEMPERATURE TREATMENTS[1]

By F. H. Peto[2]

Abstract

An apparatus was developed for applying heat treatments to spikes on plants growing in the field. This apparatus gave very satisfactory results on self-fertilized zygotes of Marquis wheat. Treatments at 42, 43, and 44° C. for 20 min. induced chromosome doubling in 2% of the plants.

A wide variety of temperature treatments was applied to more than 13,000 wheat florets 16 to 27 hr. after being pollinated with *A. glaucum*, in an attempt to produce fertile and stable F_1 hybrids. Chromosome doubling was induced in one F_1 plant of Kharkov × *A. glaucum* by exposure to alternating temperatures of 36° and 109° F. The resulting 84-chromosome plant grew slowly and failed to produce any spikes, as was the case with a number of 42-chromosome plants of this cross. Consequently there is uncertainty as to whether this plant is inherently abnormal aside from chromosome doubling, or whether the chromosome number is too high for normal development. It must be concluded from the results as a whole, that chromosome doubling by means of temperature treatments can be induced only with great difficulty in *Triticum–A. glaucum* hybrids.

Introduction

A. glaucum ($2n = 42$) crosses readily with both tetraploid and hexaploid species of wheat, but the F_1 generation is completely sterile, although backcrosses to the wheat parent have been made (1, 4). Since the F_1 plants of several of these crosses exhibit very desirable forage qualities, the problem of inducing fertility has received considerable attention.

Previously reported cytological studies (8) have shown that partial homology exists between one set of chromosomes from each of the parents with usually less than seven bivalents found at first metaphase. In addition, the chiasmata frequency per bivalent was found to be much lower in the hybrids than in the male parent *A. glaucum*, a situation that indicates a lower degree of homology among the pairing chromosomes in the hybrids. The meiotic irregularities caused by random behavior of numerous univalents are undoubtedly largely responsible for pollen degeneration and failure of anther dehiscence. However, if the chromosome number in these hybrids could be doubled, each of the chromosomes would then have a homologue with which it could pair. This should result in normal meiotic behavior and thereby overcome the main obstacle to fertility. Examples of the fortuitous doubling of the chromosome number in sterile F_1 hybrids are numerous. Winge (12) mentions 24 cases in which fertile and constant species hybrids have originated through doubling of the chromosome number. Since fertile and constant intergeneric hybrids have origininated through chromosome doubling of sterile

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Contribution from the Division of Biology and Agriculture, National Research Laboratories, Ottawa. This contribution forms part of a co-operative investigation on the hybridization of Triticum and Agropyron, undertaken by the Dominion Experimental Farms and the National Research Council of Canada.

[2] *Cytogeneticist, National Research Laboratories, Ottawa.*

F_1 hybrids such as *Aegilops ovata* ($2n = 28$) × *Triticum durum* ($2n = 28$) and *Triticum vulgare* ($2n = 42$) × *Secale cereale* ($2n = 14$), it is reasonable to expect similar results from doubling the chromosome number of *Triticum–A. glaucum* hybrids.

Randolph (10) was one of the first workers to utilize heat treatments in producing new tetraploid strains. In 1932 he was successful in inducing chromosome doubling in maize by applying heat locally to ears of corn at the time of the first zygotic division. The chief advantage of Randolph's method was that if doubling occurred at this stage the resulting plants were completely tetraploid. The author's interest in the possibilities of utilizing heat treatments to induce chromosome doubling originated from studies begun in 1933 on the influence of temperature on the mutation rate in barley (7) when tetraploid sectors in root tips of barley were observed after heat treatments of germinating barley seed. In one case a partially tetraploid barley spike was discovered in a plant that had been subjected to a temperature of 35° C. for seven days during germination (9). Attempts to duplicate these results were unsuccessful and Randolph's time of treatment was finally adopted. The effectiveness of heat treatments during the early zygotic divisions in inducing completely tetraploid plants has been recently demonstrated by several workers. Atwood (2) reported in 1936 the occurrence of a fertile tetraploid plant of *Melilotus alba* from local heat treatment of the racemes at 40 to 41° C. for 30 min. Müntzing, Tometorp and Mundt-Petersen (6) found nine barley plants with chromosome numbers from 27 to 30 in the second generation after the local application of heat treatments at 45° C. for 30 min. Dorsey (3) was successful in inducing chromosome doubling in *Triticum vulgare*, *Secale cereale* and in sterile F_1 hybrids of *T. vulgare* × *S. cereale*. The amphipolyploid wheat-rye plant was partially fertile. The meiotic behavior of this plant was not investigated but it is reasonable to assume that normal bivalent formation would occur.

Apparatus and Methods

An apparatus for applying heat treatments in the field was built and three units in operation are shown in the photograph in Plate I, Fig. 1. The exterior of the water jacket is 18 in. high and 5½ in. in diameter and constructed of 16-gauge copper. The treatment chamber proper is constructed from a 14-gauge brass tube 1½ in. in diameter and 16 in. long. This tube was soldered in position in the centre of the outer chamber with the lower end left open so that the apparatus can be slipped over the spike to be treated. A ½-in. brass tube 3 in. in length was soldered to the upper end of the 1½ in. tube, so that the bulb of a sensitive thermometer could be placed in close contact with the spikes during treatment. A DeKhotinsky thermoregulator was used to control the temperature of the water in the water jacket. Heat was applied by resistance wire wound around the lower third of the jacket's outer surface, which was suitably insulated with asbestos tape. Heat losses to the exterior were reduced by ½-in. felt held in place by cotton lagging. The

apparatus was supported on a tripod having a large base 5½ in. in diameter with a 2½ in. hole in the centre to allow the spikes to slip into the inner tube of the heater. Tests were made on the temperature variations within the treatment chamber with the bottom open. The air temperature in the portion of the treatment chamber 4 in. or more above the opening could be maintained within ±0.5° C. of the desired temperature of 40 to 48° C. In field tests it was found that two spikes could be treated at the same time without any significant depression of the air temperature at the beginning of treatment.

In 1936, the heat treatment apparatus was used exclusively on plants growing in the field, but in 1937 the experiments were modified to include low- as well as high-temperature treatments, since Sax (11) obtained much better results by this method. The plants to be hybridized and treated were grown in the field in 6-in. pots, which in turn were embedded in soil. Growth under these conditions resembled closely that of plants growing free in the soil and it was possible to remove without damage the potted plants for the duration of the treatment. The low-temperature treatments were carried out by transfer of the plants to a refrigerator room at 36° F. The high temperature treatments were carried out in small greenhouse rooms in which the temperature could be controlled within ±1° F. at any required temperature between 95 and 109° F. A very high humidity was maintained in these rooms by spraying the floors and moistening the peat on the benches prior to treatment.

The method of emasculating the wheat florets and pollination with *A. glaucum* was similar to that described by Johnson and McLennan (5). The various treatments were begun 16 to 27 hr. after pollination, with narrower limits in certain experiments.

Heat Treatments of Marquis Wheat

The objects of these preliminary treatments were to determine the optimum conditions for inducing chromosome doubling in the early zygotic divisions after natural self pollination, and to determine the effects of the various treatments on fertility. Twenty-seven spikes with 50% or more florets in flower were selected. The central florets were removed and the total number of florets reduced to 20. The time of flowering was noted, and nine spikes were treated after elapsed periods of 16, 20 and 24 hr., respectively. In each case, three spikes were heat-treated for 20 min. with the field heat treatment apparatus at each of the temperatures 42, 43, and 44° C.

The data from this experiment are given in Table I. The percentage seed set was decreased with increase in treatment temperature and with decrease in the period between pollination and treatment. These results indicate that the treatment temperatures, duration of treatments and time lapse after flowering were in the desired critical range in which chromosome doubling might be expected without complete inhibition of seed formation.

From the heat-treated zygotes of Marquis, 295 plants were grown and carefully examined during early growth. Ten plants were noticeably different

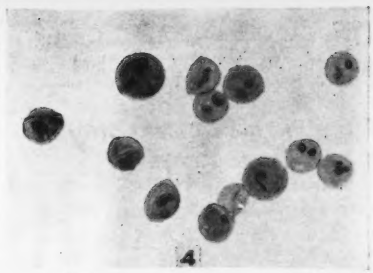

FIG. 1. *Apparatus for heat treatment in operation.* FIG. 2. *84-chromosome plant of Marquis wheat.* FIG. 3. *42-chromosome plant of Marquis wheat.* FIG. 4. *Pollen from 84-chromosome plant of Marquis wheat.* FIG. 5. *Pollen from 42-chromosome plant of Marquis wheat.*

from the others. They had a slower growth rate, the leaves appeared to be coarser and sometimes irregular in shape, and the stomata seemed larger than normal. Chromosome counts were made on nine of these plants; six plants had 84 chromosomes or double the normal chromosome number, and the remainder had the normal complement of 42 chromosomes. The 84-chromosome plants grew more slowly than the 42-chromosome plants, reaching the flowering stage about a month later under winter greenhouse conditions.

TABLE I

RESULTS FROM HEAT TREATMENTS OF MARQUIS WHEAT

Time after flowering, hr.	Temp., °C.	No. florets pollinated	No. seeds set	Seed set, %	Seed set, mean %
16	42	60	35	58.3	
16	43	54	22	40.7	46.5
16	44	60	24	40.0	
20	42	56	31	55.4	
20	43	58	36	62.0	57.0
20	44	58	31	53.4	
24	42	60	49	81.7	
24	43	60	36	60.0	66.1
24	44	60	34	56.7	

Percentage seed set at 42° = 65.1
Percentage seed set at 43° = 54.2
Percentage seed set at 44° = 50.3

They, however, eventually attained normal height, tillered abundantly and seemed slightly stouter and coarser than the normal plants. All the plants were sterile, as the anthers failed to dehisce. Pictures of 84- and 42-chromosome plants are shown in Plate I, Figs. 3 and 4. Chromosome doubling is usually disadvantageous in a highly fertile pure species with a high polyploid chromosome number, consequently the unfavorable results obtained with the 84-chromosome plants were not surprising. The experiment was, however, successful as a further demonstration of the practicability of the heat treatment method for inducing autopolyploid forms, and gave encouragement to the efforts to induce chromosome doubling in sterile *Triticum–A. glaucum* hybrids.

Comparison of pollen grains in 42- and 84-chromosome wheat plants

Ripe anthers were fixed in absolute alcohol, three parts, and glacial acetic, one part, and transferred to 70% alcohol. They were mounted and stained in aceto-carmine. Measurements were taken with a micrometer eyepiece, one unit of the recorded measurement being 15 μ. The data given in Table II show that the pollen of the 84-chromosome plant was about 5.5μ larger on the average. The values given for the standard deviation of the mean show that the pollen of the 84-chromosome plant was much more variable. The

reasons for this situation were apparent when pollen development was studied. The data on these studies are given in Table III, and pollen photographs are shown in Plate I, Figs. 4 and 5. All the pollen grains of the 42-chromosome form were normal in that the vegetative and two sperm nuclei were always present and the grains were filled with cytoplasm. Development of pollen in the 84-chromosome plants was very irregular or had been retarded at various stages. For example, only 48.5% of the grains possessed three nuclei and an equal percentage possessed only vegetative and generative nuclei. There was a reduced amount of cytoplasm in 30% of the pollen grains of this plant, and 11.5% contained only a slight amount of cytoplasm. The anthers failed to dehisce on account of these abnormalities, and the 84-chromosome plants were therefore completely sterile.

TABLE II

MEASUREMENTS OF POLLEN DIAMETER

——	Mean diameter (μ)	S.D. of the mean
T. vulgare (42-chromosome plant)		
Sample 1	56.9	0.40
Sample 2	56.1	0.48
	Mean 56.5	
T. vulgare (84-chromosome plant)		
Sample 1	60.4	0.65
Sample 2	63.4	1.02
	Mean 61.9	

TABLE III

POLLEN DEVELOPMENT IN THE 42- AND 84-CHROMOSOME FORM OF *T. vulgare*

Plants	Cytoplasm	Nuclei				Total	Per cent
		3	2	1	0		
Normal $2n = 42$	Normal	200	0	0	0	200	100
	Reduced	0	0	0	0	0	0
	Slight	0	0	0	0	0	0
	None	0	0	0	0	0	0
	Total	200	0	0	0		
	Per cent	100	0	0	0		
Mutant $2n = 84$	Normal	58	50	3	0	111	55.5
	Reduced	24	35	1	0	60	30.0
	Slight	11	10	2	0	23	11.5
	None	4	2	0	0	6	3.0
	Total	97	97	6	0		
	Per cent	48.5	48.5	3.0	0		

Stomatal measurements

The average over-all length and width of the guard cells of the leaves from 42- and 84-chromosome plants are given in Table IV. The mean stomatal

length and width of the 84-chromosome plant exceed those of the 42-chromosome plant by 9.5 and 4.5μ respectively. These differences represent increases of about $\frac{1}{5}$, whereas increases of about $\frac{1}{4}$ in linear dimensions would be expected if chromosome doubling had doubled cell volume. The differences between the plants showed up more clearly in the product of the length and width which should give a single value bearing a close relation to volume.

TABLE IV

COMPARSION OF STOMATAL SIZES IN 42- AND 84-CHROMOSOME PLANTS

Plant	Chr. no. ($2n$)	No. stomata examined	Mean length (μ)	Mean width (μ)	Mean product L × W (sq. μ)
Marquis	42	100	70.0	30.9	2,174
Marquis	84	100	79.5	35.4	2,836
F_1 Kharkov × *A. glaucum* (121–4)	42	50	59.9	33.9	2,036
F_1 Kharkov × *A. glaucum* (121–5)	42	50	52.4	33.8	1,773
*F_1 Kharkov × *A. glaucum* (121–1)	84	100	65.2	43.5	2,840
*F_1 Kharkov × *A. glaucum* (121–1)	84	50	57.4	40.8	2,347

* *Individuals of the same clone.*

If accurate estimates of volume could have been secured, it seems probable that the differences would have been even more pronounced. Considering, however, the small mean differences between the linear dimensions of the 42- and 84-chromosome plants and the wide variation between individual measurements, it is apparent that any superficial stomatal observations would be of little value in detecting 84-chromosome plants.

Temperature Treatments to Obtain Amphipolyploid *Triticum-A. glaucum* Hybrids

Experiments during 1936

A total of 5140 florets of the various tetraploid and hexaploid species and varieties of *Triticum* (Table V) were pollinated with *A. glaucum* and subjected to heat treatments with the portable apparatus 18 to 27 hr. later. The three apparatuses were set at temperatures of 42, 43 and 44° C. and the duration of treatment was 20 min. in each case. A summary of the results is given in Table V. The data for the three treatment temperatures are combined in the summary, as there were no clear-cut differences. From the 5140 florets treated, only 111 seeds were obtained, seed set in individual crosses varying from 0 to 5.5%. The germinability of the seed was rather low, as only 50 seedlings were obtained. Chromosome counts made on the root tips of these seedlings showed all to possess the undoubled chromosome number.

A partial explanation of the failure to double the chromosome number is undoubtedly the fact that only approximately 1% of the fertilized florets produced viable hybrid plants. Assuming that the frequency of induced

chromosome doubling observed in self-fertilized Marquis (2%) would apply to the hybrids, then approximately 2% of the plants from treated zygotes would be expected to have the doubled chromosome number. Consequently

TABLE V

SUMMARY OF 1936 HEAT-TREATMENT RESULTS FROM CROSSES BETWEEN *Triticum* AND *A. glaucum*

Female parent	No. florets treated	No. seeds	Per cent seed set	No. seedlings
T. vulgare var. Lutescens	1780	36	2.0	10
T. vulgare var. C.A.N. 1835	1060	7	0.7	0
T. vulgare var. Marquis	600	17	2.8	4
T. vulgare var. Canus	280	0	0	0
T. durum var. Mindum	840	46	5.5	33
T. turgidum	340	2	0.6	2
T. persicum var. Black Persian	180	3	1.7	1
T. dicoccum var. Vernal	60	0	0	0
Totals	5140	111	2.2	50

it would be necessary to treat over 5000 florets to expect even one hybrid plant with the doubled chromosome number. Since only slightly more than this number were treated, the failure in this year's experiments to obtain amphipolyploid seedlings is not surprising.

Experiments during 1937

The heat-treatment technique employed in 1936 was modified in 1937 to include alternating low and high temperatures. A wide variety of treatments was applied as shown in Tables VI and VII. It was impossible to have enough florets of any combination ready for treatment at one time to give a completely satisfactory test of any given treatment condition, since the percentage success of any treatment, as regards chromosome doubling, was expected to be very low, judging from the results of previous experiments. It was thought, therefore, that the chances of producing an amphipolyploid hybrid would be greater if a wide range of conditions were tried than if one or two treatment conditions were arbitrarily selected and thoroughly tested.

The winter wheat varieties, Dawson's Golden Chaff and Kharkov, and the spring wheat varieties, Lutescens, Marquis and Vernal emmer, were used as the female parents and pollinated with *A. glaucum*. The method of procedure in a typical treatment was as follows: The plants on which the florets had been pollinated the previous day were removed from the field at 9 a.m. and placed in the cold room at 36° F. At 9 p.m. that night these plants were transferred to the greenhouse room at 95° F. At 9 a.m. the following day they were replaced in the cold room at 36° F. and removed at 9 p.m. that night.

In certain of the treatments the field heat-treatment apparatus was also used. Following these treatments the plants were, in several experiments, transferred to the cold room at 36° F. for 10 hr.

TABLE VI

SUMMARY OF 1937 TEMPERATURE-TREATMENT RESULTS FROM CROSSES BETWEEN WHEAT AND *A. glaucum*

Temperature treatment, °F.	Female parent	Leaf and stem injury after treatment	No. florets pollinated	No. of seeds		Hybrid seed set, %	No. plants
				Prob. hybrids	Prob. selfs		
3 hr. 36°	Dawson's G.C.	0	392	11	2	2.8	7
+ 3 hr. 104°	Marquis	2	20	0	0	0	0
+ 6 hr. 36°	Lutescens	1–3	60	0	0	0	0
+12 hr. 104°	Vernal	0–1	140	1	4	0.7	0
+ 3 hr. 36°							
			612	12	6	2.0	7
3 hr. 36°	Dawson's G.C.	0–1	168	26	0	15.5	18
+ 3 hr. 109°	Kharkov	0–1	224	37	0	16.5	5*
+ 3 hr. 36°	Marquis	1–4	140	0	0	0	0
+ 3 hr. 109°	Lutescens	2–3	80	0	2	0	0
			612	63	2	10.3	23
1.5 hr. 36°	Dawson's G.C.	1	28	0	0	0	0
+2.5 hr. 111°	Kharkov	1	56	0	0	0	0
+8 hr. 36°	Lutescens	2	20	1	0	5.0	0
	Vernal	0–1	140	20	0	14.3	15
			244	21	0	8.6	15
6 hr. 36°	Dawson's G.C.	1	112	1	0	0.9	1
+ 6 hr. 104°	Marquis	0–1	80	0	0	0	0
+12 hr. 36°	Vernal	1–2	60	0	0	0	0
			252	1	0	0.4	1
12 hr. 36°	Marquis	2–4	360	27	4	7.5	3
12 hr. 104°	Lutescens	3–4	300	17	0	6.0	9
	Vernal	3–4	120	0	0	0	0
			780	44	4	5.8	12
12 hr. 36°	Dawson's G.C.	0	96	0	0	0	0
+12 hr. 95°	Marquis	0–1	280	34	0	12.1	2
+12 hr. 36°	Lutescens	0–1	520	28	11	5.4	4
	Vernal	0–1	180	70	0	38.9	57
			1076	132	11	12.3	63
6 hr. 36°	Marquis	0–1	120	50	0	41.7	11
+6 hr. 104°	Lutescens	0–1	140	66	0	47.1	15
	Vernal	0–1	180	92	0	51.1	75
			440	208	0	47.3	101
6 hr. 36°	Marquis	0–1	40	0	0	0	0
+4 hr. 104°	Lutescens	1–2	80	7	0	8.7	2
	Vernal	0–1	80	23	7	28.7	21
			200	30	7	15.0	23

**The 84-chromosome plant was found in this lot of plants.*

TABLE VI—*Concluded*

SUMMARY OF 1937 TEMPERATURE-TREATMENT RESULTS FROM CROSSES BETWEEN WHEAT AND *A. glaucum*—*Concluded*

Temperature treatment, °F.	Female parent	Leaf and stem injury after treatment	No. florets pollinated	No. of seeds		Hybrid seed set, %	No. plants
				Prob. hybrids	Prob. selfs		
6 hr. 36° +2 hr. 104°	Dawson's G. C	1	56	0	0	0	0
	Marquis	0–1	40	11	0	27.5	3
	Lutescens	1–2	60	7	0	11.7	7
	Vernal	0	20	1	0	5.0	1
			176	19	0	10.7	11
6 hr. 36° +90 min. 105°	Marquis	1	40	1	0	2.5	0
	Lutescens	0–1	60	1	0	1.7	1
			100	2	0	2.0	1
6 hr. 36° +60 min. 105°	Marquis	1	20	2	0	10.0	0
	Lutescens	0–2	100	6	6	6.0	6
			120	8	6	6.7	6
6 hr. 36° +30 min. 105°	Marquis	1	40	0	0	0	0
	Lutescens	1	40	0	0	0	0
	Vernal	0	40	7	2	17.5	7
			120	7	2	5.8	7
6 hr. 36° +90 min. 104°	Marquis	0–1	80	2	0	2.5	0
	Lutescens	1	40	2	7	5.0	0
			120	4	7	3.3	0
6 hr. 36° +60 min. 104°	Marquis	1	180	29	0	16.1	7
	Lutescens	0–1	160	5	1	3.1	1
			340	34	1	10.0	8
6 hr. 36° +30 min. 104°	Marquis	0	20	6	0	30.0	0
	Lutescens	0–1	100	19	1	19.0	2
			120	25	1	20.8	2
12 hr. 36° +12 hr. 95°	Marquis	0–4	400	10	2	2.5	1
	Lutescens	0–3	180	3	0	1.7	0
			580	13	2	2.2	1

When the whole plant was subjected to high temperatures, the more severe treatments caused extensive injury to the leaves and exposed leaf sheaths. Notes on this injury were taken and the plants graded from 0 to 5, the higher grades indicating the more severe injury. The range of injury for each treatment is given in Table VI. Foliage injury did not always inhibit seed development, since a number of hybrid seeds were set following severe injury from treatment at 104° F. for 12 hr.

TABLE VII

Temperature treatment		Female parent	No. florets	No. seeds		Hybrid seed set, %	No. plants
Field apparatus, °C.	Cold room, °F.			Prob. hybrids	Prob. selfs		
20 min. 42°	10 hr. 36°	Dawson's G.C.	112	0	0	0	0
20 min. 46°	10 hr. 36°	Dawson's G.C.	104	0	0	0	0
			216	0	0	0	0
20 min. 42°	10 hr. 36°	Marquis	220	12	0	5.5	1
20 min. 43°	10 hr. 36°	Marquis	240	6	0	2.5	0
20 min. 44°	10 hr. 36°	Marquis	60	7	0	11.7	0
20 min. 45°	10 hr. 36°	Marquis	40	0	0	0	0
20 min. 46°	10 hr. 36°	Marquis	100	0	0	0	0
			660	25	0	3.8	1
20 min. 42°	10 hr. 36°	Lutescens	120	3	1	2.5	0
20 min. 43°	10 hr. 36°	Lutescens	160	3	0	1.9	0
20 min. 46°	10 hr. 36°	Lutescens	100	5	2	5.0	3
			380	11	3	2.9	3
20 min. 42°	10 hr. 36°	Vernal	80	4	0	5.0	4
20 min. 43°	10 hr. 36°	Vernal	20	1	0	5.0	1
20 min. 46°	10 hr. 36°	Vernal	60	0	0	0	0
			160	5	0	3.1	5
20 min. 43°		Marquis	60	0	0	0	0
20 min. 44°		Marquis	20	0	0	0	0
			80	0	0	0	0
20 min. 42°		Lutescens	20	2	0	10.0	0
20 min. 45°		Lutescens	120	0	0	0	0
20 min. 46°		Lutescens	140	0	0	0	0
20 min. 48°		Lutescens	40	0	0	0	0
			320	2	0	0.6	0
20 min. 42°		Vernal	20	0	0	0	0
20 min. 44°		Dawson's G.C.	140	0	1	0	0
20 min. 45°		Dawson's G.C.	84	0	0	0	0
			224	0	1	0	0
Untreated check		Dawson's G.C.	28	1	0	3.6	1
Untreated check		Kharkov	28	1	0	3.6	0
Untreated check		Marquis	60	4	1	6.7	2
Untreated check		Lutescens	140	4	0	2.9	0
			256	10	1	3.9	3

The most striking feature of the results recorded in Tables VI and VII is the lack of correlation between severity of treatment and amount of seed set. However, the treatments are not strictly comparable as it was impossible to pollinate enough plants to enable more than two or three treatments to

be carried out simultaneously. Consequently the following variables might influence the results: (1) stigmatic receptivity; (2) viability of pollen; (3) influence of weather on pollen tube growth rate.

The seed set following heat treatment with the apparatus in the field was lower than that for similar heat treatments carried out in the greenhouse. For example, the apparatus treatment at 42° C. (107.6° F.) for 20 min. would be expected to be less severe than two three-hour periods at 109° F. in the greenhouse. However, the amount of seed set from Dawson's Golden Chaff pollinated with *A. glaucum* was 15.5% after the latter treatment, and none was set after the former. The additional severity of the apparatus treatments may be the result of very rapid heat transfer from contact of the spike with the inner surface of the metal tube, and also because of the lower humidity of the air surrounding the spike.

TABLE VIII

POLLINATION, SEED, AND GERMINATION DATA FOR VARIETIES OF WHEAT POLLINATED WITH *A. glaucum* AND SUBJECTED TO VARIOUS TEMPERATURE TREATMENTS

Wheat variety	No. florets pollinated	No. hybrid seeds	Hybrid seeds, %	No. plants grown	Viability, %
Dawson's G.C.	1292	38	2.9	27	71.1
Kharkov	280	37	13.2	5	13.5
Marquis	2600	197	7.6	30	15.2
Lutescens	2640	176	6.7	50	28.4
Vernal (emmer)	1140	219	19.2	181	82.6
Total	7952	667	8.4	293	43.9

A total of 7952 wheat florets were pollinated with *A. glaucum* and subjected to various temperature treatments (Table VIII); 667 seeds were produced and 293 plants were grown. The viability of the seed of Dawson's Golden Chaff and Vernal hybrids was good, while that of the other hybrids was very poor. The percentage of hybrid florets producing seeds after temperature treatment was similar, on the average, to the results obtained by Armstrong (1) and Johnson (4) without heat treatment, with the exception that Johnson reports a much higher seed set for Dawson's Golden Chaff hybrids than that obtained in the present study. There is some evidence that moderate temperature treatments may greatly increase the hybrid seed set, because after a treatment of 6 hr. at 36° F. and 6 hr. at 104° F. the seed set for Marquis, Lutescens, and Vernal hybrids was 41.7, 47.1 and 51.1%, respectively. Johnson's results (4) for the same crosses were only 2.0, 2.5 and 6.4%, and Armstrong's results were 8.4% for Lutescens and 34.6% for Vernal hybrids.

Chromosome counts were made on the root tips of most of the 293 hybrid plants, which with one exception were found to possess the undoubled chromosome number. This exceptional plant was from a cross between Kharkov and *A. glaucum* and possessed 84 instead of 42 chromosomes. The treatment producing this amphipolyploid plant was one of the most severe applied.

It began about 18 hr. after pollination and was as follows: 9 to 12 a.m. at 36° F., 12 to 3 p.m. at 109° F., 3 to 6 p.m. at 36° F., and 6 to 9 p.m. at 109° F. A total of 224 florets were treated in this way and 26 hybrid seeds were obtained, from which five plants were grown. Two of these plants were very weak; one died in the seedling stage, and the other was so puny that root-tip counts could not be made. Chromosome counts were, however, made on the other three plants, two of which had 42 chromosomes and the other, as indicated, 84 chromosomes. One of the 42-chromosome plants (121–5) was rather slow growing and failed to produce any spikes, as was the case with the 84-chromosome plant, although the latter was slightly more vigorous. The other 42-chromosome (121–4) plant was very vigorous, produced an abundance of sterile spikes and in general resembled the better F_1 plants of this cross produced in previous years.

The failure of the 84-chromosome plant to produce any spikes prevented a meiotic study being made to determine whether chromosome doubling would result in normal bivalent formation. There are two possibilities that might account for the failure of this plant to develop normally. Either it is inherently abnormal, regardless of chromosome doubling, as is the case with a number of the 42-chromosome plants of this cross, or the chromosome number is too high for normal growth and differentiation. If the former is the case, it would be worth the effort to produce other hybrids with the doubled chromosome number. However, it should be kept in mind that the 84-chromosome wheats did not develop normally. It is therefore likely that better results might be expected from doubling the chromosome number in crosses between the tetraploid ($2n = 28$) wheats and *A. glaucum* which would give an amphipolyploid variety with 70 chromosomes.

A study was made on the relation of cell size in root tips to chromosome doubling. Comparable cross sections of the root tips of the 84-chromosome hybrid plant and of two 42-chromosome plants of the same cross were examined. The outlines of the areas in which the cell size was to be determined were drawn with the aid of a *camera lucida* and the area measured with a rotometer. The area of the cross section studied was a sector comprising one-quarter of the circumference which included the epidermal and the three outer endodermal layers. The number of cells in an area was counted and the average area per cell calculated. The approximate cell volume was calculated by cubing the square root value of the mean cell area. The data are shown in Table IX and clearly indicate that cell volume in root tips is directly proportional to chromosome number. The ratio in cell volume of 42- and 84-chromosome plants would be expected to be 0.5, whereas actual calculations showed it to be 0.525 and 0.559.

Stomatal sizes were also compared in the 42- and 84-chromosome F_1 plants of Kharkov × *A. glaucum* (Table IV). The same plants were studied as were used for the cell-size measurements in root tips, but the leaves were collected on one-year-old plants and the root tips were studied in the seedling stage. The cell size in the root tips was very similar in plants 121–4 and 121–5, but the stomatal size of the former was much larger. Plant 121–4 is much

more vigorous than 121–5 and this may partially account for the larger stomatal size. This, however, does not explain the differences in stomatal size between individuals of the same clone of the 84-chromosome plant (121–1) which should be equally vigorous. It is suspected that the slightly different stages of development at which the leaves were collected might account for this lack of agreement. In spite of the above variation, the mean product of stomatal length and width of the 84-chromosome individuals is greater than that of the 42-chromosome individuals. These data substantiate the conclusions drawn from observations on Marquis regarding the unreliability of stomatal measurements as a positive indicator of chromosome doubling.

TABLE IX

COMPARISON OF CELL SIZE BETWEEN 42- AND 84-CHROMOSOME HYBRIDS OF KHARKOV × *A. glaucum* (Magnification = 350×)

Plant No.	Chromosome number	Sector area, sq. in.	No. of cells	Cell area, sq. in.	Calculated cell volume, cu. in.	Ratio 42 chr./ 84 chr.
121–5	42	2.61	30	0.087		
		2.80	30	0.093		
		2.46	27	0.091		
		2.43	26	0.093		
			Average	0.091	0.0275	0.525
121–4	42	2.60	27	0.096		
		2.59	27	0.096		
		2.34	25	0.094		
		2.56	27	0.095		
			Average	0.095	0.0293	0.559
121–1	84	3.85	27	0.143		
		3.72	27	0.138		
		3.82	26	0.143		
		3.74	28	0.134		
			Average	0.140	0.0524	

Discussion

Heat treatments at 42, 43, and 44° C., applied to Marquis wheat 16 and 24 hr. after self-pollination, with the specially constructed heat-treatment apparatus caused the doubled chromosome number to occur in about 2% of the resulting plants. This method is therefore very satisfactory for inducing chromosome doubling in fertile, non-hybrid zygotes. However, when this method is applied to hybrid zygotes in which the percentage seed set is very low, the chances of inducing chromosome doubling are greatly reduced, as is seen from the treatments in 1936 of 5140 florets with negative results. Consequently, if chromosome doubling is to be induced by temperature treatments of a moderate number of hybrid zygotes, either the treatment methods must be improved, so that a chromosome doubling would occur in a much higher percentage of hybrid zygotes, or the present methods restricted

to hybrid combinations in which a high percentage seed set might be expected. In the 1937 experiments, high and low temperature treatments were alternated in the hope that this might prove a more effective method and, in addition, certain wheats were selected which were expected to set a high percentage of seed on being pollinated with *A. glaucum*. In spite of these improvements only one amphipolyploid plant was produced from 7952 florets pollinated. Peculiarly enough, this plant was produced under very severe treatment conditions in which only 224 florets of Kharkov pollinated with *A. glaucum* had been treated. It is possible that duplication of this treatment on a large scale on hybrid zygotes which set a high percentage of seed might yield satisfactory results. Nevertheless, it must be concluded that chromosome doubling by means of temperature treatments can be induced only with great difficulty in *Triticum–A. glaucum* hybrids.

The alkaloid colchicine has recently been found to be particularly useful in doubling chromosome numbers in plants. Studies in this laboratory have shown it to be much more effective with dicotyledonous than with monocotyledonous species. Nevertheless, the possibilities of using this drug to obtain amphipolyploid *Triticum–A. glaucum* hybrids is being thoroughly tested before further temperature treatments are undertaken.

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BACTERICIDAL AND ANTIGENIC QUALITIES OF THE WASHINGS OF BLOWFLY MAGGOTS[1]

BY RONALD GWATKIN[2] AND A. MURRAY FALLIS[3]

Abstract

Washings of mixtures of maggots of *Calliphora erythrocephala*, *C. latifrons*, *C. vomitoria nigribarda* and *Cynomya cadaverina*, and of *C. erythrocephala* alone, showed bactericidal activity against *Staph. aureus*, *Strep. mastitidis*, two strains of *Brucella abortus*, and *B. typhosus in vitro*. Flies were successfully raised in the laboratory but washings from their maggots became less active with each generation. The pH value of active samples was generally greater than that of poor samples. Attempts to increase activity by rendering poor washings alkaline were unsuccessful. Diluted washings showed some bactericidal power. Keeping quality of liquid samples was poorer than that of dried ones. Washings from meat in which maggots had been grown had a definite bactericidal value, while those from control samples of meat had none. A suspension of maggots ground after washing was also inactive. Addition of *Br. abortus* to meat on which maggots were grown did not increase bactericidal activity against this organism. Filtration reduced bactericidal action and toxicity. Intraperitoneal injections were toxic to guinea pigs. An anti-maggot rabbit serum had good complement fixing power with an antigen of maggot washings. Intraperitoneal injections of diluted washings failed to protect guinea pigs against infection with *Br. abortus*, but there was some delay in development of infection, as indicated by slower appearance of agglutinins in treated animals.

Introduction

Baer (1) in 1929, and in subsequent papers, reported on the successful use of blowfly maggots in surgical conditions.

Simmons (6) in 1935 obtained a bactericidal substance from the excretions of surgical maggots, *Lucilia sericata*. The active principle was not destroyed by autoclaving for 20 min. at 10 lb. pressure. Simmons points out that the perfection of a non-toxic, non-irritating disinfectant for internal use has not yet been realized and he suggests there are possibilities in this type of material.

Robinson (5) in 1935 reported the isolation of allantoin from maggot excretions. This substance is a product of metabolism of the cell nucleus of both plants and animals, and its excretion into wounds by the maggots is apparently one of the factors contributing to the healing effect in maggot therapy.

Stewart (7) in 1934 found that maggots of *L. sericata* exuded calcium carbonate. One hundred maggots produced an average of 0.6 mg. every 24 hr. He recommends treatment of osteomyelitis with calcium carbonate,

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Contribution from the Ontario Research Foundation, Toronto, Canada.

[2] *Formerly Research Fellow, Ontario Research Foundation; now Director, Veterinary Research Station, Lethbridge, Alberta.*

[3] *Research Fellow.*

picric acid, and glycerin. Frost and Errington (3) have found that this was beneficial in post-operative treatment of fistulous withers in horses.

It is curious to note, on the one hand, the beneficial effects of maggot therapy, and on the other, the harmful results produced by the same kind of maggots under natural conditions, an example of which is seen in the serious economic loss caused by blowflies in the sheep industry in Australia, Britain, and other sheep-raising countries. However, it is obvious that a substance possessing bactericidal qualities and capable of stimulating healing is of more than ordinary interest, if these qualities are not offset by others, such as toxicity, that might prevent its parenteral use.

Methods and Materials

Method of Raising Flies

A culture of flies was established by attracting them to a dead rat or guinea pig, catching them in a net and enclosing them with pieces of raw beef in cheesecloth-covered glass jars. The flies that hatched from eggs laid on the meat provided stock cultures. The original flies were identified by Dr. C. H. Curran, Assistant Curator, Department of Entomology, New York Museum of Natural History. They were *Calliphora erythrocephala* Mg., *C. latifrons* Hough, *C. vomitoria nigribarda* S., and *Cynomya cadaverina* Desv.

The flies were kept in cages 2 ft. by 2 ft. by 2 ft., enclosed by cheesecloth, in a room about 19° C. The temperature was frequently lower and occasionally higher. The flies were fed on sugar and fresh meat. Water was supplied by inversion of a beaker on a Petri dish covered with a filter paper. An electric light close to the cages appeared to increase activity of the flies.

Larvae for experimental purposes were obtained by leaving a dish of meat in a cage of flies for several hours. When the meat was well "blown", the dish was removed to a glass jar that was covered with cheesecloth. The meat was covered with moist absorbent cotton to keep it from drying, as room humidity was low. After one or more washings to collect the experimental material, the larvae were allowed to pupate in dry sand.

The time intervals between the various stages of *C. erythrocephala*, although showing considerable variation due to inconstant temperature, were roughly as follows: eggs hatched in from 10 to 20 hr.; larval period lasted four to six days; prepupal period was one to three days, and the pupal stage about two weeks. The flies after emerging from the pupae were not ready to deposit eggs until a week or more later.

A relatively high humidity appeared to be favorable for all pre-adult stages, but the metamorphosis into pupae appeared to be favored by relatively dry conditions. Insufficient humidity during the pupal stage causes hardening of the cases and may prevent emergence of the flies.

Preparation of Experimental Material

Maggots were removed from the meat about four days after hatching and placed in an 80-mesh sieve on which another sieve was fitted to prevent their

escape. Removal of maggots was facilitated by using a relatively small amount of meat. Saline solution (0.85% NaCl) was dropped on them at regular intervals over a period of two hours, until a quantity had been used equal to one-half the volume of the maggots. The washings thus obtained were a dark green, or brownish-green, turbid liquid. This was autoclaved at 10 lb. pressure for 20 min., tested for sterility and stored in the refrigerator. Dried samples were not autoclaved until made into suspensions for use.

Bactericidal Tests

Bactericidal tests were carried out after the manner of phenol coefficient tests. The procedure was similar to the method of Simmons (6). The results confirm those obtained by Simmons, although different kinds of bacteria were used as well as different species of maggots. Quantities of 2.5 cc. of sterilized washings were placed in test tubes in a water bath at 37° C. Twenty-four-hour broth cultures of bacteria were employed, 0.05 cc. of which was added to each tube at one-half minute intervals and also to the same quantity of salt solution for each culture against which the suspension was being tested. A standard loopful was removed from each tube of washings at 2.5, 5, 15, 30, and sometimes at 60 min. Transfers from the saline tubes were usually made after the conclusion of the experiment, but in some tests were made at the same time-intervals as the suspension under test. The broth tubes to which transfers had been made were incubated for 48 hr. at 37° C. *Staphylococcus aureus*, *Streptococcus mastitidis*, *B. typhosus* and two strains of *Br. abortus* were used at various times. There was a marked difference in resistance of the two *Brucella* strains. No. 10, an old laboratory strain, was much more easily killed than a more recently isolated culture. Suspensions were usually tested the day they were prepared, but in a few instances on the following day.

Results

Bactericidal Activity of Fresh Samples

Washings were obtained from 16 lots of maggots. Most of the lots were made up of more than one species of larvae, but some were prepared from the pure strain of *C. erythrocephala*. The source of the maggots, and the effect of the washings on the bacteria against which they were tried, are shown in Table I.

It will be noticed that better results were obtained from the early lots than from those produced from larvae of laboratory-raised flies. We have been unable to determine the cause of this. It was thought that crowding the maggots during the washing process might have been responsible, as a large quantity of maggots was placed in the sieve in the later experiments, completely filling it. This possibility was investigated with Lots 13 and 13A, which consisted of larvae from the same meat divided into small and large lots. There was no difference in bactericidal activity, but the larger lot had a slightly lower pH value. Mr. W. C. Henry, of the Department of Biochemistry, determined the pH of the maggot washings and saline solution

TABLE I

RESULTS OF BACTERICIDAL TESTS OF MAGGOT WASHINGS

Lot	Maggots	pH of washings	Staph.	Strep.	*Br. abortus*		Typhoid
					No. 10	Page	
1	A, B and C	–	5	2.5	–	–	–
2	A	10.9	5	2.5	2.5	–	15
2A	A, 2nd washing	–	15	2.5	2.5	–	15
3	A, and D	9.6	2.5	2.5	2.5	15	30
4	A, 1st generation	10.0	–	–	–	5	–
5	B and C, 1st gen.	8.7	X	15	15	30	30
6	A, 1st gen.	8.5	–	–	2.5	5	–
7	B and C, 1st gen.	5.7	X	30	15	X	X
8	A, 1st gen.	7.9	30	2.5	–	–	–
9	A, B and C, 1st gen.	9.4	15	–	5	–	–
10	B and C, 1st gen.	7.1	–	–	30	30	–
11	B and C, 1st gen.	7.6	–	–	30	30	–
12	A, B, C and D, 1st gen.	6.3	X	30	–	–	–
13	A, B, C, D, mostly 2nd gen.	6.7	X	15	X	–	–
13A	A, B, C, D, mostly 2nd gen.	7.2	X	15	X	–	–
14	A, 3rd gen.	5.6	X	X	–	–	–
15	A, 3rd gen.	4.6	–	X	–	–	–
16	A, 3rd gen.	4.9	–	X	–	–	–
	Saline	5.8	X	X	X	–	–

Figures represent time in minutes at which transfers were sterile. X—growth from 30-min. tubes. Not examined further. A—Calliphora erythrocephala Mg. B—Calliphora latifrons Hough. C—Cynomya cadaverina Desv. D—Calliphora vomitoria nigribarda S.

TABLE II

COMPARISON OF UNDILUTED AND DILUTED SAMPLES

Washings		Strep.	*Br. abortus*	
			No. 10	Page
Lot 1	—	2.5	–	–
Lot 1	1 : 100	60	–	–
Lot 2	—	2.5	2.5	–
Lot 2	1 : 100	30	30	–
Lot 2A	—	–	15	15
Lot 2A	1 : 10	–	30	30
Lot 2A	1 : 50	–	X	X
Lot 2A	1 : 100	–	X	X
Lot 4	—	–	5	5
Lot 4	1 : 10	–	30	30
Lot 4	1 : 20	–	30	30
Lot 4	1 : 50	–	XX	X
Lot 4	1 : 100	–	XX	X
Lot 6	—	–	5	5
Lot 6	1 : 10	–	30	30

Figures represent time in minutes at which transfers were sterile. X—growth from 30-min. tubes. XX—growth from 60-min. tubes.

(0.85% NaCl) by means of a double hydrogen electrode. Lots 2 to 11 and 14 to 16 had been kept in liquid form. The others had been dried and were resuspended in distilled water. The results are given in Table I. It will be noticed that the samples with higher pH values are, generally speaking, more bactericidal than those with a lower pH. Lot 5 appears to be the only exception. Lots 14, 15 and 16, prepared from the maggots of third generation flies, were the poorest of all, showing no bactericidal activity in 30 min. against the most easily killed organism, *Strep. mastitidis*. Lot 16 still showed growth after 60 min.

Although the various samples were not tested against the three organisms in all cases, there is sufficient information to show clearly the superiority of the earlier samples. It was noticed in the later samples that the color of the washings was not as dark and the suspensions did not appear as turbid.

Bactericidal Activity of Diluted Samples

A comparison was made of washings undiluted and diluted up to 1 : 100 with saline solution against *Strep. mastitidis* and two strains of *Br. abortus*. The results are shown in Table II.

The bactericidal activity rapidly disappeared on dilution at the time intervals employed. The suspensions were not tested after 60 min. and in some cases 30 min., as indicated in the table.

Effect of Suspending Dried Washings in Alkaline Solutions

In view of the results noted in connection with the pH reactions of the samples, dried washings from Lot 13 were suspended in a boric acid-potassium chloride-sodium hydroxide buffer solution and in Sørenson's glycocoll-sodium chloride-sodium hydroxide buffer solution, which were made after the method of Clark (2). Other suspensions were made in alkaline salt solution. Germicidal tests were made with these various solutions. There was a suggestion from the results of these tests that a high pH value is connected with bactericidal activity, although it may only be incidental, but on the whole the results were rather indefinite.

Loss in Activity of Dried and Liquid Samples

Samples were tested after subjecting them to various conditions. These results are given in Table III. The advantage of storage in the dried rather than in the liquid state is evident.

TABLE III

Comparison of fresh and old samples

Lot	Age	Bactericidal action			
		Staph.	Strep.	Brucella	Typhoid
2	Fresh	5	2.5	–	–
	22 days (dried)	15	2.5	–	–
	80 days (dried)	30	2.5	–	–
2A	Fresh	15	2.5	2.5	15
	7 days (liquid)	30	30	15	X
3	Fresh	2.5	2.5	–	–
	22 days (dried)	15	2.5	–	–
5	Fresh	X	15	15	–
	7 days (dried)	X	15	30	–
	7 days (liquid)	–	30	30	–

Figures represent time in minutes at which transfers were sterile. X—*Growth from 30-min. tubes.*

The samples were poured on to a sheet of glass about 9 in. above a hot plate and dried in the draught of a fan. The material was scraped off and stored in vials at room temperature. The amount of dry material varied somewhat with different lots, the percentage yield of Lots 2, 3, 5, 9, 12, 13, and 13A being 1.8, 2.0, 1.3, 2.2, 1.4, 1.6, and 1.6, respectively. It should be remembered that 0.85% salt solution was used for washing the maggots and the salt is included in these weights. The dried material was brought back to the original volume with distilled water when required and was autoclaved at that time. There was no apparent relation between percentage of solids and bactericidal activity of the washings.

Comparison of Maggots, Beef from Which They Were Taken and Non-maggoty Beef

Maggots from Lot 2, meat in which they had been raised, and a piece of the same meat not exposed to flies but kept under the same conditions, were washed with equal amounts of saline solution for a period of two hours. The washings were tested against *Staph. aureus*, and *Strep. mastitidis*. The results are shown in Table IV.

TABLE IV

Bactericidal test of maggots, maggoty and non-maggoty meat

Washings	Bacteria	Exposure in minutes			
		2.5	5	15	30
Maggots	Staph.	+	−	−	−
Maggoty meat	Staph.	+	+	−	−
Control meat	Staph.	+	+	+	+
Maggots	Strep.	−	−	−	−
Maggoty meat	Strep.	−	−	−	−
Control meat	Strep.	+	+	+	+

+ = *growth in tubes of broth at 48 hr. after exposure indicated.*
− = *sterile after exposure indicated.*

The meat in which the maggots had grown contained the bactericidal substance, whereas meat without maggots, kept under the same conditions, showed no trace of this substance.

Comparison of Bactericidal Activity of Maggot Washings and a Suspension of Ground Maggots

Maggots (50 cc. in volume) from Lot 1 were washed, then ground with sand in 50 cc. saline. The mixture was filtered through coarse paper, autoclaved at 10 lb. for 20 min., and tested for sterility. The washings and the ground suspension were tested at the same time for bactericidal activity against *Staph. aureus*. Transfers were made at 2.5, 5, 15 and 30 min.; those from washings were sterile in 5 min. The suspension of ground maggots showed no bactericidal power. Growth was obtained from seedings made from the suspension the following morning.

Attempt to Increase Bactericidal Activity Against *Br. abortus* by the Addition of this Organism to the Meat

An attempt was made to determine whether growth on specifically infected meat would increase the activity of maggot excretions against *Br. abortus*.

A portion of meat was heavily spread with the organism, while a second was left untouched. Both were seeded with eggs of the first generation of *C. erythrocephala*. Five days later, an equal volume of maggots was collected from each. These were washed in the usual manner, and both suspensions were tested against two strains of *Br. abortus*. There was no difference between the two lots (Lots 10 and 11 in Table I), both of which were low in bactericidal activity.

Filtration of Maggot Washings

Washings were filtered through paper and a Mandler candle. The material was difficult to filter, but a small amount of clear green fluid was obtained. Lot 2 filtrate did not kill streptococci in 30 min., while the suspension did so in 2.5 min. Lot 4 filtrate was tested against *Br. abortus*. The suspension tube was sterile at 5 min. and the filtrate tube at 15 min. Both showed growth with streptococci at 5 but not at 15 min. Lot 5 filtrate did not kill staphylococci or *Br. abortus* in 30 min. but killed streptococci in 15 min. The unfiltered material did not kill staphylococci in 30 min. but killed *Br. abortus* and streptococci in 15 min. Lot 8 filtrate did not kill staphylococci in 30 min. but killed streptococci in 5 min. Lot 8 suspension killed the former in 30 and the latter in 2.5 min.

Filtration, therefore, did not remove all the bactericidal activity but lowered it. It will be seen that the same holds true in regard to toxicity.

Toxicity of Maggot Washings

Guinea pigs weighing from 350 to 400 gm. were given intraperitoneal injections of 1.0 cc. of various lots of washings and filtrates. All fresh lots caused a rapid fall in temperature, and in many cases death.

Lot 1 fresh and after 5 days in refrigerator. The temperatures of these guinea pigs are shown in Table V. The fresh material caused a rapid fall in temperature and death. In the second test there was some drop in temperature but recovery occurred rapidly.

TABLE V

Temperatures of guinea pigs injected with fresh washings and the same washings after five days in refrigerator

Sample	10:30 a.m.	11:30 a.m.	2:00 p.m.	4:00 p.m.	5:30 p.m.	9:00 a.m.
Fresh washings	102.4	96.2	94.0	94.0	94.0	Dead
Fresh washings	103.0	96.4	94.0	94.0	94.0	Dead
Old washings	103.0	102.2	101.8	101.4	102.2	103.0
Old washings	102.8	101.4	100.2	101.2	101.4	103.0

The reaction in these animals appeared to be very similar to that seen after injections of alcoholic precipitates of bacteria or fresh cow serum. There were marked signs of discomfort following the injection. The abdomen rapidly

became tense. In some cases there was a watery discharge from the eyes. The animals went off their feet in 3 or 4 hr., and death usually took place in 5 to 6 hr. Post-mortem examination showed a considerable amount of clear fluid in the abdominal cavity and intense inflammation of peritoneum. Cultures were usually sterile. In animals that died more slowly, a fibrinous exudate formed in the abdominal cavity, beginning around the edges of the liver. In a few instances, guinea pigs apparently recovered at the time but died a couple of weeks later.

Lots 4 and 8 washings and filtrate. One-cc. portions of filtered and unfiltered suspensions of each of these lots were injected intraperitoneally the day after preparation. The guinea pigs receiving unfiltered suspension showed a more rapid drop in temperature and were very ill for one to two days, whereas the animals receiving filtrate returned to normal in about one-half this time. Although none of the animals died, it was clear from their temperatures and general appearance that some of the toxic quality had been removed by filtration, just as the bactericidal activity is lowered.

Comparison of a Sample while Fresh and after Storage in Dry and Liquid Form for Three Weeks

One portion of a toxic sample was kept in the refrigerator for three weeks. Another portion was dried and resuspended three weeks later, while a third was injected intraperitoneally into a guinea pig the day after preparation. The other two samples were subsequently injected in the same manner. Table VI gives the temperatures of three guinea pigs injected with 1.0 cc. of the suspensions.

TABLE VI

Temperatures of guinea pigs

Sample	Time			
	3:00 p.m.	4:00 p.m.	5:00 p.m.	10:00 a.m.
Fresh washings	101.8	94.0	94.0	Dead
Liquid 3 weeks old	102.4	102.0	101.0	103.0
Dry 3 weeks old	102.0	98.2	94.0	102.8

There was a loss of toxicity in both samples that had been held for three weeks, but it was more pronounced in that which had been stored in liquid form.

Antigenic Quality of Maggot Washings

A rabbit was given five intravenous injections of a dried sample of washings over a period of 10 days. Blood was collected from the heart 10 days after the last injection, and sterile serum was prepared from it. This was employed in the complement fixation test and also tested for protective action in guinea pigs against a dried sample of washings.

Complement fixation test. The same lot of dried washings used for the production of the above serum was brought to its original concentration in water, autoclaved, and diluted 1 : 100, 1 : 1000 and 1 : 10,000 with saline solution. A sample was also heated at 56° C. for 30 min., in place of auto-

claving, and gave the same result as the autoclaved sample. Diluted antigen (1 cc.) was employed and undiluted serum was added to make dilutions of from 1 : 25 to 1 : 1000. Two units of complement were used. The hemolytic mix consisted of three units of a 1 : 6000 dilution of hemolysin in 0.25 cc. and 0.25 cc. of 2.5% sheep cells. The result of this test is shown in Table VII.

TABLE VII

COMPLEMENT FIXATION TEST WITH MAGGOT WASHINGS AND ANTI-MAGGOT SERUM

Antigen dilution	Serum	Serum dilution						Antigen alone	Serum alone		
		1 : 25	1 : 50	1 : 100	1 : 250	1 : 500	1 : 1000		1 : 25	1 : 50	1 : 100
1 : 100	Anti-maggot	4	4	4	4	4	4	0	0	0	0
1 : 1000	Anti-maggot	4	4	4	4	0	0	0	–	–	–
1 : 10,000	Anti-maggot	0	0	0	0	0	0	0	–	–	–
1 : 100	Normal	0	0	0	0	0	0	0	0	0	0

4 = *complete fixation;* 0 = *complete hemolysis.*

The suspension when diluted 1 : 100 and 1 : 1000 made good antigen with this anti-maggot serum. No fixation of complement occurred with the 1 : 10,000 dilution. The experiment also showed that the washings were capable of producing a good complement-binding serum. Normal rabbit serum produced no fixation in combination with these antigen dilutions.

BACTERICIDAL TESTS *in Vivo*

Experiments were carried out with the object of protecting guinea pigs against infection by intraperitoneal injections of maggot washings, which had been sufficiently diluted to avoid the toxic action of the substance. *Br. abortus* was used for the infecting agent because in previous work this organism had proved to be very consistent in infecting guinea pigs. The work was carried out in a manner similar to that employed in the anti-abortus serum experiments of the senior author (4).

The guinea pigs were infected in some experiments by injecting a light suspension of *Br. abortus* intraperitoneally, in others by placing a drop of the suspension in the eye. Some of the guinea pigs in each group were then given daily injections of dilutions of 1 : 5 to 1 : 50 of maggot washings. Different dilutions were used in different experiments and the time of commencing treatment with them was varied.

There was a delay in agglutinin production in the treated animals, but they were all found to be infected on post-mortem examination.

ACTION OF MAGGOT WASHINGS ON GUINEA PIG ERYTHROCYTES *in Vitro*

Four lots of dried washings were brought to their original concentration and autoclaved in the usual manner. These, and six additional fluid samples, were added to portions of a 1% suspension of washed guinea pig red cells. Cell suspension (1 cc.) was placed in small tubes and to these 0.01, 0.1 and

1.0 cc. of each sample were added. The tubes were then incubated for one hour in the water bath at 37° C. The results are shown in Table VIII.

TABLE VIII

ACTION OF MAGGOT WASHINGS ON GUINEA PIG RED CELLS *in vitro*

Lot	State	0.01 cc.	0.1 cc.	1.0 cc.
5	Fluid	No change	Partial hemolysis	Partial hemolysis
6	Fluid	Hemolysis	Hemolysis	Hemolysis
7	Fluid	Agglutination	Agglutination	Hemolysis
8	Fluid	Hemolysis	Hemolysis	Hemolysis
10	Fluid	Agglutination	Agglutination	Hemolysis
11	Fluid	Agglutination	Hemolysis	Hemolysis
2	Dried	Agglutination	Hemolysis	Hemolysis
3	Dried	Hemolysis	Hemolysis	Hemolysis
5	Dried	Agglutination	Hemolysis	Hemolysis
9	Dried	Agglutination	Partial hemolysis	Hemolysis

It was rather difficult to determine what had taken place in the 0.1 and 1.0 cc. tubes, on account of the dark color of the washings. A heavy precipitation was noted in both tubes when hemolysis had occurred. A rather clear, green supernatant fluid remained. The sediment did not contain blood cells. Evidently the cells were hemolyzed and the hemoglobin was quickly precipitated. The smallest dose usually agglutinated the red cells without any hemolysis, but in two instances complete hemolysis occurred in these tubes. It will be noticed that Lot 5 fluid was not as active as the dried sample of this lot. Both were examined 18 days after preparation.

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ANTI-*BRUCELLA* SERUM: GUINEA PIG PROTECTION EXPERIMENTS[1]

By Ronald Gwatkin[2]

Abstract

Guinea pigs were protected against infection with *Brucella abortus* by intraperitoneal injections of fresh, unpreserved, anti-abortus rabbit serum. In the first two experiments the serum-treated and control animals were exposed to infection by contact with infected guinea pigs, while in the third experiment they were exposed by instillation of the infecting organism into the eye. In the first experiment 60% of the controls and none of the treated animals were infected. In the second, 80% of the controls and 20% of the treated animals were infected. In the third, all of the treated animals were protected, while all of the controls became infected. The former method, although less certain than the latter, is worthy of consideration in experiments of this type, as it permits infection to occur in a natural manner.

Introduction

In previous papers the writer (2–4) reported that repeated injections of the serum of immunized guinea pigs and rabbits protected normal guinea pigs against infection with *Brucella abortus* by mouth and eye. Serum did not prevent the development of infection in animals infected prior to receiving injections of serum. Cow and horse serum were much less satisfactory in prophylactic experiments with guinea pigs.

The work of Beach (1) confirmed our results with cow serum in guinea pigs. He found that the protective power of such serum for these animals was not very high.

Mitchell, Humphreys and Walker (5) found that anti-*Brucella* horse serum conferred a degree of protection when administered to guinea pigs previous to a challenge inoculation of *Br. abortus*, but if administered after infection was established it did not confer any protection.

In view of our results with anti-abortus rabbit serum in the protection of guinea pigs against exposure to *Br. abortus* by mouth and eye, it was decided to study the effects of injecting such serum into animals exposed to infection by contact with artificially infected guinea pigs. Such an exposure would be less severe than the artificial methods employed in the previous experiments, but might also be expected to be less certain.

Experiment 1. Prevention of Natural Infection

Preparation of serum. Ten large rabbits were given four weekly injections of an old strain of *Br. abortus*. Growth from the flat surface of beef agar in three 16-ounce medicine bottles was collected (after three days' incubation at 37° C.) in 25 cc. of salt solution, and 0.5 cc. was injected intraperitoneally the first time, followed by injections of 2.0 cc. Thirty-five days later these rabbits were given an intraperitoneal injection of 0.5 cc. of a light suspension

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[2] *Research Fellow.*

of a recently isolated strain of *Br. abortus* (No. 2 McFarland nephelometer). A week later the growth from two liver agar slants was collected in 20 cc. of salt solution, and the rabbits were given intraperitoneal injections of 2 cc. of this suspension. These injections were continued throughout the experiments.

About 20 cc. of blood was drawn from the heart of each rabbit once a week and the blood was allowed to clot in sterile vials. The withdrawal of two lots of 10 cc. with a short interval between operations was found to be safer than withdrawing 20 cc. at one time. The serum was collected aseptically and stored without preservative in the refrigerator until used. It was usually injected the same week in which it was collected. In this way a much greater quantity of serum was obtained than would have been possible if the rabbits had been sacrificed for serum collection, and fresh unpreserved serum was available for each injection. Guinea pigs would have been more satisfactory as sources of serum, as serum injections could have been given to the experimental animals after longer intervals. Guinea pigs are much less resistant to *Br. abortus* than rabbits, and the mortality during serum production is high. Agglutination tests were carried out with blood obtained each week prior to the injections of *Br. abortus*. After the first rise to 1 : 10,000 in many of the rabbits, the titre of all varied between 1 : 1000 and 1 : 2500 with an occasional rise in some to 1 : 5000.

Experimental guinea pigs. Thirty-two guinea pigs, one group of seven and five groups of five, were used in this experiment. Seven were exposed to infection by eye with one drop of a suspension of *Br. abortus*. Seven days later the untreated group and the four serum-treated groups were placed in the same pen. Intraperitoneal injections of 2 cc. of serum were given to the treated groups at various intervals. The groups were as follows:

Group 1. Infected; 3 males and 4 females.
Group 2. Untreated; 3 males and 2 females.
Group 3. Serum 3 times a week; 3 males and 2 females.
Group 4. Serum twice a week; 3 males and 2 females.
Group 5. Serum once a week; 2 males and 3 females.
Group 6. Serum every 2 weeks; 2 males and 3 females.

Three of the last group, as might have been expected, died from anaphylactic shock, as did also one of Group 5. The two groups were then combined and given serum once a week.

The strain of *Br. abortus* employed to infect the guinea pigs of Group 1 was not very virulent, although recently isolated, because at 29 days only one showed agglutinins. The controls (Group 2) were negative at this time. Group 1 was then injected intraperitoneally, and two animals died from peritonitis the following day. A week later the five survivors of this group were positive by the agglutination test. Failure of the first exposure to produce infection prolonged the experiment and necessitated giving more serum injections than would otherwise have been required. The groups

were bled a week later, at which time the sera of the animals in Group 1 had agglutinin titres of 1 : 1000 or more, while the controls were still negative. At the fifth weekly bleeding one control was found to be positive, a second was found positive at the sixth week, and a third at the seventh week. The other two remained negative.

On April 13, three months from the beginning of the experiment, the last injection of serum was given and the groups were placed in separate cages. For some undetermined reason a few of the serum animals died after removal from the pen to cages. There was no intercurrent infection and we were fortunately able to determine the absence of any evidence of *Brucella* infection. Details of these animals are given later. The serum guinea pigs were all positive at this time but subsequently ceased to react, while the control group and the infected group remained unchanged, *i.e.*, three positive and two negative in the control and all positive in the infected group. They were killed on May 12, blood was collected for the agglutination test, and cultures were made. The results follow.

Group 1 (Infection only). Five guinea pigs. All were emaciated, two lost the use of their hind limbs, spleens of all were enlarged and nodular, lesions in testes of the two males. Agglutinin titres ranged from 1 : 250 to 1 : 1000. Cultures from four yielded *Br. abortus*.

Group 2 (Controls). Five guinea pigs. Three were positive by the agglutination test and two were negative. The spleens of the former were enlarged and *Br. abortus* was recovered from them on culture. The latter showed no lesions and cultures remained sterile.

Group 3 (Serum three times a week). Five guinea pigs. Two males died about 10 days after being placed in cages. In both cases the gastric mucous membrane was covered with punctate haemorrhages, which could also be observed under the serous coat. No other changes except emaciation were noted. The spleens of these animals were normal in appearance, cultures were negative and only a trace of agglutination at 1 : 25 remained. Their serum titres had previously been 1 : 100 and over, owing to injections of serum. The other three animals were negative at the time they were killed, spleens were normal and cultures remained sterile.

Group 4 (Serum twice a week). Five guinea pigs. One male died about the same time as the two in Group 3. It showed the same condition of the gastric mucous membrane. The serum still had a slight agglutinative power shortly before death, due to the injections of positive serum, but post-mortem findings and cultures showed no evidence of infection. The other four animals were negative when they were killed, spleens were normal and cultures negative.

Group 5 (Serum once a week). One of this group of five guinea pigs died early in the experiment from anaphylactic shock. One of the remaining four died May 2 and the same gastric lesions were observed as in those that died of Groups 3 and 4. No agglutinins were demonstrated in the blood,

the spleen was normal in appearance and cultures were sterile. One male and two females survived. They were negative by the agglutination test, spleens were normal and cultures negative. These females each had one young guinea pig several weeks old at the conclusion of the experiment. The young animals were also shown to be free from infection by the agglutination test, cultures, and absence of lesions.

Group 6 (Originally serum every two weeks but changed to once a week). Three of the five animals in this group died of anaphylactic shock after the second injection of serum, and the survivors were then given serum once a week as in Group 5. These animals showed no agglutinins at the time they were killed, spleens were normal in appearance and cultures were negative.

Results of Experiment 1

In view of the fact that intra-abdominal injections of serum were given to Groups 3 to 6 from one to three times a week, no particular importance could be attached to the birth of young in this experiment. In all the serum groups some females carried their young to full term, in spite of the injections, but the young animals, with the exception of those in Group 5, died after a few days from lack of nourishment as the females had little or no milk. There was no difference in protection in the groups receiving one or three injections each week. Three of the five control guinea pigs became infected (60%). Four of the serum animals died at the beginning from anaphylactic shock. None of the remaining 16 showed any evidence of infection at the conclusion of the experiment. There would have been a more clear-cut picture if all the controls had become infected, but under natural conditions of infection this could hardly be expected. None of the serum-treated animals became infected and it is quite plain that they were completely protected under conditions that caused the infection of 60% of the control group.

Experiment 2. Prevention of Natural Infection

In the first experiment there did not appear to be a sufficiently large group of infected animals to cause a rapid spread of infection to the controls. In the present experiment this was made the largest group. One group of serum-treated animals and a control group of the same size were used. Injections of rabbit serum were given twice a week to avoid the danger of anaphylactic reactions.

Preparation of serum. Serum from the rabbits used in Experiment 1 was used for protective injections. Weekly bleedings and injections of *Br. abortus* were continued as formerly, and more rabbits were immunized in the same manner, but, as sufficient serum was available from the first lot, these were not bled until the thirteenth injection on May 26.

Experimental guinea pigs. Thirty-five guinea pigs were used. Group 1 consisted of 15 animals, which were infected by intraperitoneal injection. Group 2 consisted of 10 untreated controls, and Group 3 of the remaining 10, which were injected with serum. All were placed in the same pen on April 14,

at which time Group 1 was infected and Group 3 received the first injection of serum. Groups 1 and 2 were bled at weekly intervals starting the second and third weeks after infection, and were killed June 16, nine weeks after the beginning of the experiment. Particulars of these groups are given under their respective headings.

Group 1 (Infection only). Fifteen guinea pigs infected by intraperitoneal injection of a suspension of *Br. abortus* on April 14. One female died of peritonitis seven days after injection. The organism was recovered in pure culture. All the others appeared ill but recovered, although a few continued to drag their hind legs. The 14 survivors were bled two weeks after injection and the agglutination test was set up to 1 : 200, at which dilution all sera showed complete agglutination. There were nine males and five females. These animals had gained only an average of 55 gm. each when they were killed on June 16. The spleens of all were considerably enlarged, the testes of the males were atrophied but were not abscessed. None of the females were pregnant. *Br. abortus* was recovered from 13 of the 14. Four had an agglutinin titre of 1 : 500 and ten of 1 : 1000 or over.

Group 2 (Controls). Ten untreated guinea pigs, seven of which were males. They were weighed, properly identified and placed in the same pen as Group 1. They were bled on May 5, three weeks after the beginning of the experiment, and at weekly intervals thereafter. The first test showed two males and one female were infected. The number gradually increased, as shown in Table I, and on June 16, when they were killed, eight were infected.

TABLE I

AGGLUTINATION TESTS ON GROUP 2 (CONTROLS)

No.	Sex	May				June		
		5	12	19	26	2	9	16
1	M	—	—	—	—	—	—	—
2	M	1 : 25	1 : 25	1 : 100	1 : 100	1 : 100	1 : 250	1 : 500
3	F	1 : 25	1 : 100	1 : 100	1 : 100	1 : 100	1 : 250	1 : 100
4	M	1 : 100	1 : 100	1 : 250	1 : 500	1 : 1000	1 : 1000	1 : 1000
5	M	—	—	—	—	1 : 50	1 : 100	1 : 250
6	F	—	—	1 : 25	1 : 25	1 : 50	1 : 100	1 : 250
7	F	—	1 : 50	1 : 100	1 : 100	1 : 250	1 : 100	1 : 100
8	M	—	—	—	—	—	—	—
9	M	—	—	—	—	—	—	1 : 100
10	M	—	—	—	—	1 : 50	1 : 100	1 : 100

Nos. 1 and 8 (Table I) were found to be normal on post-mortem examination, and cultures were negative. The spleens of all the others except No. 9 were enlarged, and *Br. abortus* was recovered from all except No. 3, cultures of which were overgrown with mold. No. 6 was pregnant and *Br. abortus* was recovered from the foetal membranes in addition to the spleen. This group had made an average gain of 131 gm. at this time.

Group 3 (Serum twice a week). Ten guinea pigs, of which six were males. They were given an intraperitoneal injection of 2 cc. of serum on April 14, and twice a week thereafter, until a total of 19 injections had been given; the last was given on June 16, at which time eight of the controls were positive to the agglutination test. They and the animals of Group 1 were killed, while those of Group 3 were placed in clean quarters after receiving the last injection of serum. On this date the animals of this group showed an average gain of 212 gm. each. Agglutination tests were not carried on during the serum injection period but were begun on the day of the last injection, when the serum titres ranged from 1 : 50 to 1 : 100. Results of these tests are shown in Table II.

TABLE II
AGGLUTINATION TESTS ON GROUP 3 (SERUM-TREATED GUINEA PIGS)

No.	Sex	June			July
		16	23	30	6
1	M	1 : 100	1 : 50	1 : 25	—
2	M	1 : 100	1 : 50	—	—
3	F	1 : 50	1 : 25	1 : 25	—
4	M	1 : 100	1 : 25	—	—
5	M	1 : 50	1 : 200	1 : 100	1 : 100
6	M	1 : 50	1 : 25	1 : 25	—
7	M	1 : 50	1 : 50	—	—
8	F	1 : 100	1 : 100	1 : 100	1 : 100
9	F	1 : 50	1 : 25	—	—
10	M	1 : 50	1 : 25		—

The guinea pigs of Group 3 were killed on July 7. Post-mortem examination showed all to be normal except Nos. 5 and 8. The former had an enlarged spleen and in the latter the spleen was large, nodular and somewhat haemorrhagic. This animal showed an early pregnancy. The other two females were also pregnant, No. 9 being practically at full term. Cultures from all were negative but the presence of lesions and persistence of agglutinins in Nos. 5 and 8 clearly indicate that these two animals were infected.

Results of Experiment 2. The strain of *Br. abortus* used in this experiment was evidently quite satisfactory, judged by the appearance of lesions in Groups 1 and 2.

Groups 1 and 2 were killed on June 16, at which time serum injections for Group 3 were stopped, and the animals were placed in clean quarters, where they remained until killed on July 7. On June 16 the average gain in weight of the original infected group was 55 gm., that of the control group 131 gm., and that of the serum-treated group 212 gm.

All the animals in Group 1 were infected. Eight of the 10 untreated controls became infected (80%) while only two of the serum-treated group showed evidence of infection at the conclusion of this experiment (20%).

EXPERIMENT 3. PREVENTION OF INFECTION BY EYE

While Experiment 2 was in progress a small group was treated along the lines reported in earlier papers, to which reference has been made. This was done in order to have a direct comparison between the severity of natural exposure and artificial exposure by eye, with the same serum as used in Experiments 1 and 2 and the same culture as used in Experiment 2.

Experimental guinea pigs. Two groups of three guinea pigs were employed. Each animal in Group 1 was given 2 cc. of serum intraperitoneally on May 9. Group 2 was left untreated. The following day both groups were exposed to infection by eye with one drop of a suspension of the same strain of *Br. abortus* as used in the previous experiment. The suspension was of such a density that when diluted with three parts of saline it was equal to Tube No. 1 of the McFarland nephelometer. Six injections of 2 cc. of serum were given to Group 1 over a period of three weeks and both groups were bled for the agglutination test and weighed once a week. Agglutination results are given in Table III.

TABLE III

AGGLUTINATION RESULTS IN EXPERIMENT 3

Group	No.	Sex	May				June				July
			9	16	23	30	6	13	20	27	4
I	1	F	—	1 : 100	1 : 50	1 : 50	1 : 25	1 : 25	—	—	—
	2	M	—	1 : 100	1 : 100	1 : 50	1 : 50	1 : 25	—	—	—
	3	F	—	1 : 100	1 : 100	1 : 100	1 : 100	1 : 100	1 : 100	1 : 50	--
II	1	F	—	—	1 : 25	1 : 100	1 : 100	1 : 100	1 : 100	1 : 100	1 : 100
	2	F	—	—	1 : 25	1 : 100	1 : 100	1 : 100	1 : 100	1 : 100	1 : 100
	3	F	—	—	1 : 50	1 : 100	1 : 100	1 : 100	1 : 100	1 : 100	1 : 100

All were negative at the beginning of the experiment and prior to the injection of serum. The following week the serum animals of Group 1, as would be expected, were positive, while the untreated ones had not yet developed agglutinins, because of infection. These were first observed two weeks after the beginning of the experiment.

The serum-treated group lost 60 gm. the first week as compared with 20 gm. in the control group. When taken from the pens and placed in wire-bottomed cages guinea pigs always show a temporary loss in weight, such as occurred in Group 2. In Group 1 the greater loss in weight can only be ascribed to the serum injections. Intraperitoneal injections of almost any substance will cause similar losses in weight. Both groups showed a loss in weight at the conclusion of the experiment, the loss in Group 1 still being the greater, namely, 42 gm. against 13 gm. in Group 2.

On May 26 the last injection of serum was given to Group 1. Weekly bleedings and weighings were continued until July 4, when all the serum-treated animals were negative and both groups were killed. There were no gross lesions in either group but cultures from Group 2 were positive while those from the serum-treated group were negative.

Results of Experiment 3. The serum-treated group was protected against eye infection by six injections of serum. Agglutinins had disappeared at the conclusion of the experiment, and cultures were negative. The control animals showed agglutinins, and cultures yielded *Br. abortus* from all spleens

but no gross lesions were observed. Both groups lost weight, the loss being greater in the serum-treated group.

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WOOL GROWTH AND QUALITY AS AFFECTED BY CERTAIN NUTRITIONAL AND CLIMATIC FACTORS*

III. RESULTS AND CONCLUSIONS[1]

By J. E. Bowstead[2] and P. Larose[3]

The purpose of this investigation and the experimental treatment of the sheep were described in Part I (1) of the series, and the laboratory methods of testing the wool were described in Part II (2). This paper presents the results obtained by these methods, and completes the series.

Results

The differences in the performance of animals between any two experimental groups are largely dependent upon the differences in the conditions imposed.

As stated in Part I, the experimental periods in the later trials were lengthened to ensure greater significance to the results. Temperature differences varied from 23.8 to 52.2° F., owing to differences in the average outdoor winter temperatures and the temperatures that were maintained in the heated pens.

Humidity differences between Lots I and II were necessarily dependent upon the differences between outdoor and indoor temperatures, since cold winter air was brought into these two lots and was raised by an average of from 23 to 52° F. In Lot I a humidifier raised the relative humidity to the outdoor relative humidity. In Lot II, with no humidifier, the relative humidity varied from 7.4% to 31.5% less than in Lot I, which variation was due in part to yearly differences in average outdoor and indoor temperatures and to improved ventilation of pens during the last three years.

The differences between the conditions imposed on other comparable groups of ewes varied according to kind and amounts of feeds and were more readily controlled by those conducting the trials.

The differences between comparable groups were greatest in the 4th trial.

A brief summary of the differences imposed on the comparable groups is given in Table I.

Effect of Humidity

The effect of increasing humidity in heated pens did not cause any significant results. While the data for the five years show an average decrease in fibre length of $6.2 \pm 3.5\%$, the greater part of this average decrease was due to the results obtained during the first two trials when humidity differences were less than in later trials. In the fourth year, when humidity differences were the greatest, the decrease in fibre length was only $1.7 \pm 10.4\%$, which reduced the significance of the five-year average result.

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Contribution from the Department of Animal Husbandry, University of Alberta, Edmonton, and the Division of Chemistry, National Research Laboratories, Ottawa.
[2] *Associate Professor of Animal Husbandry, University of Alberta, Edmonton, Alberta.*
[3] *Chemist, National Research Laboratories, Ottawa.*
Presented in a series of three papers.

The differences in fibre diameters and tensile strength show little or no effect of humidity differences. The examination of the fleeces of the two groups at Weston also showed little or no difference in fineness or strength.

TABLE I

DIFFERENCES IN CONDITIONS IMPOSED

Trial	Av. temp. diff. between Lots I and III, °F.	Av. relative humidity diff. between Lots I and II, %	Plane of nutr'n daily TDN* fed, Lots			Per cent more protein fed to Lot VIII than to Lot VI	Amt. more mineral fed Lot IX than Lot VI daily, lb.
			V lb.	VI lb.	VII lb.		
1st	41.8	7.4	1.69	2.04	2.06	43	.012
2nd	23.8	8.2	1.95	2.11	2.30	31	.010
3rd	37.5	15.8	1.79	1.86	2.09	44	.012
4th	52.2	31.5	.91	1.41	1.89	90	.016
5th	42.0	25.4	1.19	1.56	1.65	39	.010

*Total digestive nutrients.

While the weighted average of the grease data is not significant, it may be noted that in the 4th trial, in which the largest humidity differences were obtained, the ewes in the high humidity pen produced fleeces considerably lower in percentage of grease than those produced by the ewes in the drier pen. The ewes in the high humidity pen, however, produced fleeces with a slightly greater suint content in every trial.

While the results indicate that the higher humidity condition may slightly affect certain characters of wool growth and quality, there is insufficient evidence to believe that under usual winter management practices humidity differences could be great enough to materially affect the growth of wool or its quality.

Effect of Temperature

In four out of five trials the wool produced by the ewes in the low temperature group was longer than wool produced by the ewes in the heated pen. The difference in average length of wool between the two groups was especially large in the 1st trial, when the average temperature difference was fairly large, and moderately so in the 4th trial, when the temperature difference was greatest; but there was sufficient variation in the data to reduce the significance of the results below that usually accepted for such trials.

There was also a tendency for the higher temperature to cause a reduction in fibre diameter and tensile strength. These changes, while not significant, are not contrary to the frequent observation that any retardation in wool growth is accompanied by increased fineness and fibre weakness. Differences in fineness and strength were not noted when the fleeces of the two groups were examined at Weston, where the examination was not confined to that small portion of the fibre that grew during the experimental period.

In four of the five trials the fleeces produced under high temperature conditions averaged from 3.1 to 5.4% less in clean wool. The difference in percentage of grease between the fleeces of the two groups varied both ways in the different trials, so that the difference in percentage of clean wool may have been due to dirt and suint.

From these results it could be concluded that when fair shelters and bedding are provided, there is no advantage in keeping sheep in heated pens during the winter time at temperatures such as existed during these five trials.

Effect of Early Shearing

During the first four years, the ewes that were used in both lots had been sheared at the end of the previous May or beginning of June, and therefore carried about six months' wool growth when fall samples were taken. Only samples of fleece were taken for laboratory analysis at the end of February or beginning of March from those in the "early sheared" group. Similar samples were taken from the "late sheared" group at the usual shearing time, when the entire fleeces were removed from both lots. The data are, therefore, based on early and late samples removed from the ewes, the early samples representing about nine months' fleece growth and the samples taken at the usual time from the late sheared group representing 12 months' growth.

This accounts for the fact that, for the first four years, the late sheared group have fleeces from 25 to 40% longer than the early sheared fleeces. When both groups of fleeces represented 12 months' growth, the early sheared fleeces were slightly longer, but the difference was not significant.

The significant differences between the two groups were in the diameter and tensile strength measurements. In four of the five trials the average fibre diameters and tensile strength were greater for the early sheared fleeces. There may be two possible explanations for these results. The natural tendency for some or all sheep to "shed" their wool in the early spring months is characterized by a thinning and weakening of the fibres at the growing point. When sheep are sheared late this thin and weak portion is easily detected on grading. On the other hand, when ewes are sheared early, before the "shedding" process has commenced, the thinner and weaker portion becomes the tip of the following year's fleece and is probably not as easily detected under the usual grading methods. The second explanation for the effect of early shearing may be that when sheep are sheared early they do not undergo the discomfort of carrying a heavy fleece during the warmer days of spring, which may alter the rate of growth and size of the wool fibre as it is formed in the follicle.

As previously mentioned, the data obtained during the first four years were obtained from samples removed at the start of the trials and at the different times of shearing. The entire fleeces of both groups were removed at the usual or "late" shearing time. Therefore, fleece weight data for the first four years cannot be compared. In the 5th trial the difference of 2.2 lb. in favor of the late sheared group can be shown to be chiefly due to inherited individual differences in wool production. In 1936, two years later, six of

the ewes that had been in the late sheared group in the 5th trial gave fleeces that averaged 1.1 lb. heavier than the fleeces from six of the ewes that had been in the early sheared group of the same trial, although all were exposed to identical conditions.

In every trial the early sheared fleece samples had less shrink, or in other words a greater percentage of clean wool, than the late sheared fleece samples. This would indicate that there may be an increased secretion of grease and suint between the time of "early" and "late" shearing or approximately between March 1 and June 1.

The results of the inspection of the fleeces at Weston in 1933 supported the results of the laboratory tests on the fleeces, in that the coarser fleeces were produced by sheep sheared about March 1. However, in 1934 no differences were noted. According to Mr. W. H. J. Tisdale of the Canadian Co-operative Wool Growers Ltd., earlier shorn fleeces examined at the warehouse were superior to later shorn fleeces.

Sufficient data have been presented to show that earlier shearing can be recommended as a means of strengthening the fleece and reducing the shrinkage. Delay in shearing may reduce the value of the fleece.

Plane of Nutrition

The three groups of ewes in this study, at low, medium and high planes of nutrition, were supposed to be kept on sub-maintenance, maintenance and fattening rations respectively. Owing to individual differences and to differences in amount of feed eaten (because of group feeding), there was considerable variation in the gains made by the ewes in any one group.

Prior to the first four trials the ewes had been on pasture during the entire pasture season. The 5th trial began in June after the ewes had been sheared at the end of the 4th trial. All groups, therefore, were transferred from the 4th trial into the 5th trial without being allowed the usual pasture period. Almost all the ewes became unthrifty in all lots, failed to eat their respective feed allowances and lost considerable weight. The results of the 5th trial cannot, therefore, be regarded as significant.

Significant differences in wool growth and quality were found between the fleeces produced by ewes on the low and medium plane rations, and also between the fleeces produced by the ewes on the low and high plane rations. Less significant results were secured when the fleeces of the ewes on the medium and high plane diets were compared. Such results are in accord with those of numerous workers who conclude that wool growth and quality could be materially affected only when normal thrift and health were greatly altered.

The ewes fed the medium and high plane rations produced fleeces that were significantly longer in staple and heavier both on raw and clean wool bases than the fleeces produced by the ewes on the low plane diet. There was a tendency also for the fleeces of the ewes on the higher plane diets to be greater in diameter and strength with a greater percentage of grease than the fleeces produced in the low plane lot. This heavier grease percentage may have been the cause of the greater shrink.

The results of the examination of fleeces at Weston in 1932, 1933, and 1934 corresponded favorably with the laboratory results. In general, the fleeces of the low plane group were consistently weaker with less lustre and lower yields.

It may be stated, therefore, that when ewes are fed inadequate rations and as a consequence become thin and unthrifty, wool growth and quality are adversely affected. The data would also tend to show that a normal fleece growth was secured by maintaining the ewes in a fair condition of flesh since still heavier feeding, as in Lot VII, did not stimulate a significantly greater production of wool.

Effect of Protein

Casein was chosen as the protein supplement because it contained little of other nutrient factors that are of physiologic significance, and because of its low cystine content, which might have an influence on wool growth and quality.

The amount of casein fed varied. As the casein was fed in the concentrate mixture, the difference in the protein intake and the difference in the nutritive ratio of the low and high protein rations depended on both the amount of concentrate mixture fed and amount of roughage consumed.

TABLE II

Trial	Lot VI, low protein	Lot VII, high protein	Length of experimental period, days
1st	1 : 10.0	1 : 7.0	98
2nd	1 : 9.8	1 : 7.4	143
3rd	1 : 9.2	1 : 6.7	154
4th	1 : 8.6	1 : 4.2	202
5th	1 : 9.1	1 : 6.5	370

Based on roughage and concentrate consumption, the nutritive ratio of the two rations and length of experimental periods for the two groups were as shown in Table II.

The first three trials are somewhat similar for differences in the nutritive ratio of the rations, but in the 4th trial the high protein group received 90% more protein because of a smaller hay and larger concentrate allowance.

Even in those trials where the differences in the amount of protein were greatest, or where the experimental periods were longest, the feeding of increased amounts of casein (technical) did not affect the growth or quality of wool. The individual trends for the wool characters studied were very variable throughout the five trials. The fleeces of the two groups also showed little or no difference when examined at Weston.

These results are not in agreement with those of some earlier investigators, but the results of later experiments agree with or help to explain those obtained in these trials. There may be several reasons why the negative results were secured:

(i) The ewes were mature and therefore required less protein.

(ii) Casein is an unbalanced protein, being low in cystine, and probably could not be utilized efficiently.

(iii) Casein did not contain other physiologically active substances, as contained in some protein supplements used by other investigators.

(iv) Sufficient protein may have been fed the ewes in the low protein lot to meet their requirements.

Effect of Minerals

The percentages of the mineral supplements in the grain mixtures were as follows:

1st trial—2% calcium carbonate, 1% sodium sulphate.
2nd trial—2% calcium carbonate, 1% sodium sulphate.
3rd trial—2% calcium lactate, 1% sodium sulphate.
4th trial—1½% calcium lactate, 1% sodium sulphate, 1½% mono-sodium phosphate.
5th trial—1½% calcium lactate, 1% sodium sulphate, 1½% mono-sodium phosphate.

The amount of mineral supplement consumed by the high mineral group depended on the amount of grain fed. The greatest amount consumed was in the 4th trial, when the feeding period was fairly long, grain consumption fairly high, and the percentage mineral supplement 4%.

Regardless of the length of trial, amount of supplement or kind of minerals fed, no significant effect on wool growth and quality was noted. It would appear that the basal ration of oats and Prairie hay, oat hay, or Western Rye, provided sufficient minerals for normal wool growth and quality. Had the ewes been immature or had the soils of the districts in which the feeds were grown been abnormally low in these minerals, the results might not have been the same.

Conclusions

The maintenance of sheep in a heated pen at the same relative humidity as normal winter outdoor humidity did not increase wool growth or improve quality above that produced by ewes maintained in a heated pen with a considerably lower relative humidity. This indicates that, under the usual methods of management and climatic conditions, there would be no advantage in increasing the relative humidity of heated pens in areas where low humidity conditions occur naturally.

The maintenance of sheep in heated pens during the winter months, when periods of extremely low temperatures often occurred, had little effect on wool growth and quality. This indicates that there is no advantage in keeping sheep in heated pens if adequate shelter is supplied.

Early sheared fleeces were slightly coarser and stronger than late sheared fleeces. This suggests that sheep should be sheared as early as weather conditions permit, and where suitable shelters can be provided in case of inclement weather.

The feeding of sub-maintenance rations to pregnant ewes during the winter season seriously reduced the wool growth and quality. Maintenance of the ewes in a fair condition of flesh resulted in the production of fleeces that could be regarded as normal. Heavy feeding or maintenance of ewes in a high condition of flesh slightly increased the weight of fleece (five-year average, 0.3 lb. clean wool). To obtain normal lamb crops and fleeces normal in both

weight and quality, it appears most profitable to maintain sheep in a fair condition of flesh.

Mature ewes, when fed larger quantities of a protein supplement, did not produce fleeces that were heavier or of better quality than those produced by ewes on a basal ration of non-leguminous hays and oats. The results indicate that the purchase of high-priced protein supplements is not advisable when a fair basal ration is fed.

Mature ewes, when fed non-leguminous roughages and oats plus calcium, phosphorus and sulphur-containing salts, did not produce fleeces any heavier or of better quality than ewes not fed these mineral salts. Except in areas where the soil is especially deficient in these mineral elements, there does not appear to be any practical value in feeding these mineral supplements when the ewes can be maintained in a fair condition of flesh on a non-leguminous ration. (It is essential that all sheep be fed common salt and iodine in proper quantities.)

Appendix

Mathematical Methods Used

As indicated in Part II, wool samples were taken from each sheep at the beginning and end of the experimental periods, and sufficient measurements were made on every sample for each character to secure a fairly accurate average measurement.

In this manner average figures for each animal were available for further study. For example, in the 4th trial the figures for fibre length were as follows:

Ewe No.	Nov. sample, cm.	June sample, cm.	Difference, cm.	Difference, %	d^2
Lot V					
374	5.0	9.0	4.0	80	289
379	5.0	10.2	5.2	104	49
384	4.7	8.9	4.2	89.4	57.8
385	5.1	8.9	3.8	74.5	506.2
671	4.6	9.8	5.2	113	256
675	4.3	9.5	5.2	120.9	571.2
689	6.3	12.0	5.7	90.5	42.2
700	4.6	9.7	5.1	110.9	193.2
Total	39.6	78.0	38.4		1964.6
Average	5.0	9.8	4.8	*97* Table III	
Lot VI					
361	5.0	9.5	4.5	90	1142.4
368	6.2	13.3	7.1	114.5	86.5
371	5.2	11.3	6.1	117.3	42.2
386	5.7	11.5	5.8	101.8	484.0
657	5.1	11.7	6.6	129.4	31.4
673	4.6	11.7	7.1	154.3	930.2
695	4.5	11.1	6.6	146.7	524.4
692	4.5	11.2	6.7	148.9	630.0
Total	40.8	91.3	50.5		3871.1
Average	5.1	11.4	6.3	*123.8* Table III	

TABLE III

AVERAGE PERCENTAGE INCREASES IN FIBRE LENGTH, FIBRE DIAMETER, TENSILE STRENGTH, GREASE, AND AVERAGE FLEECE YIELDS FOR THE DIFFERENT LOTS DURING THE FIVE YEARLY TRIALS

—	No. ewes	Increase in fibre length, %	SE	No. ewes	Increase in fibre diameter, %	SE	No. ewes	Increase in tensile strength, %	SE	No. ewes	Per cent increase in % grease	SE	No. ewes	Raw wool prod., lb.	Clean wool, %	Clean wool prod., lb.
Lot I. High Humidity, High Temperature																
1st trial	8	38.4 ± 5.6		8	− 3.7 ± 2.2		7	4.9 ± 4.8		7	76.8 ±13.7		8	6.7	58.4	3.9
2nd trial	8	32.4 ± 4.4		8	6.8 ± 1.9		8	− 1.5 ± 3.3		8	25.3 ± 6.0		8	8.2	61.7	5.0
3rd trial	6	65.6 ± 4.6		7	− 8.1 ± 3.0		7	−20.0 ± 4.8		7	33.4 ± 4.9		7	6.5	64.0	4.2
4th trial	7	76.1 ± 7.6		6	−10.3 ± 2.6		6	−12.5 ± 5.0		8	7.2 ± 6.8		8	8.4	44.8	3.8
5th trial	4	54.8 ± 3.6		5	3.0 ± 4.2		5	−13.5 ± 9.9		4	8.3 ±14.1		5	9.2	54.5	5.0
Average		51.9 ± 2.5			− 2.3 ± 1.2			− 7.9 ± 2.4			31.3 ± 4.1			7.7	56.6	4.3
Lot II. Low Humidity, High Temperature																
1st trial	8	47.7 ± 5.6		8	− 1.4 ± 2.2		8	1.6 ± 4.5		7	61.8 ±13.7		8	7.6	60.4	4.6
2nd trial	8	41.8 ± 4.4		8	4.3 ± 1.9		8	−11.6 ± 3.3		6	39.1 ± 6.9		8	7.6	64.7	4.9
3rd trial	8	64.6 ± 4.0		8	− 5.7 ± 2.8		8	−17.0 ± 4.5		8	30.7 ± 4.6		8	6.7	64.3	4.3
4th trial	8	77.8 ± 7.1		8	− 7.3 ± 2.2		8	−14.5 ± 4.3		8	25.0 ± 6.8		8	8.8	47.9	4.2
5th trial	4	59.1 ± 3.6		4	3.6 ± 4.8		4	8.8 ±11.1		4	21.6 ±14.1		4	9.7	58.4	5.7
Average		58.1 ± 2.4			− 1.8 ± 1.2			− 8.2 ± 2.2			36.4 ± 4.2			7.9	59.2	4.6
Lot III. Low Temperature, Early Shearing																
1st trial	8	69.1 ± 5.6		8	6.1 ± 2.2		8	4.5 ± 4.5		6	48.8 ±14.8		8	6.8	63.8	4.3
2nd trial	8	35.6 ± 4.4		8	1.8 ± 1.9		8	−10.6 ± 3.3		8	9.2 ± 6.0		8	7.3	66.1	4.8
3rd trial	8	66.7 ± 4.0		8	− 6.8 ± 2.8		8	−11.6 ± 4.5		8	43.2 ± 4.6		8	6.8	63.9	4.3
4th trial	8	91.0 ± 7.1		8	− 7.9 ± 2.2		8	−11.8 ± 4.3		8	19.6 ± 6.8		8	8.0	48.8	3.9
5th trial	4	53.7 ± 3.6		4	2.4 ± 4.8		4	11.0 ±11.1		4	15.9 ±14.1		4	8.8	57.6	5.1
5th trial	4	11.0 ± .6*		—	—		—	—		4	30.2 ± 4.2**		—	—	—	—
Average		65.6 ± 2.7***			− 1.2 ± 1.2			− 5.3 ± 2.2			29.0 ± 4.1***			7.4	60.3	4.4

* *Average fibre length in cm. at end of 5th trial.*
** *Average percentage grease to clean wool in fleeces at end of 5th trial.*
*** *Average of first 4 years.*

TABLE III—*Continued*

AVERAGE PERCENTAGE INCREASES IN FIBRE LENGTH, FIBRE DIAMETER, TENSILE STRENGTH, GREASE, AND AVERAGE FLEECE YIELDS FOR THE DIFFERENT LOTS DURING THE FIVE YEARLY TRIALS—*Continued*

—	No. ewes	Increase in fibre length, %	SE	No. ewes	Increase in fibre diameter, %	SE	No. ewes	Increase in tensile strength, %	SE	No. ewes	Per cent increase in % grease	SE	No. ewes	Raw wool prod., lbs.	Clean wool, %	Clean wool prod., lbs.
Lot IV. Low Temperature, Late Shearing																
1st trial	7	101.4 ± 5.9		7	4.0 ± 2.4		7	− 1.9 ± 4.8		6	64.1 ±14.8		7	6.9	49.1	3.4
2nd trial	7	62.6 ± 4.7		7	2.2 ± 2.0		7	−30.6 ± 3.5		7	18.0 ± 6.4		7	7.2	65.4	4.7
3rd trial	7	106.7 ± 4.2		7	− 8.5 ± 3.0		7	−18.1 ± 4.8		6	31.2 ± 5.3		7	7.5	62.8	4.7
4th trial	8	126.1 ± 7.1		8	−13.9 ± 2.2		8	−10.0 ± 4.3		8	33.3 ± 6.8		8	9.9	45.1	4.5
5th trial	6	10.2 ± .5*		7	− 6.7 ± 3.6		7	− 4.5 ± 8.4		4	32.5 ± 4.2**		4	11.0	57.0	6.3
Average		100.1 ± 2.9***			− 4.8 ± 1.2			−12.9 ± 2.4			35.7 ± 4.5***			8.3	55.5	4.6
Lot V. Low Plane of Nutrition																
1st trial	7	67.3 ± 5.9		8	− 1.4 ± 2.2		8	7.2 ± 4.5		7	49.5 ±13.7		8	7.1	57.6	4.1
2nd trial	8	42.0 ± 4.4		7	− 1.3 ± 2.0		8	−12.7 ± 3.3		8	14.7 ± 6.0		8	7.3	64.0	4.6
3rd trial	8	60.6 ± 4.0		8	− 6.1 ± 2.8		8	− 2.5 ± 4.5		8	39.8 ± 4.6		8	6.0	69.5	4.2
4th trial	8	97.0 ± 7.1		8	−14.1 ± 2.2		8	−25.2 ± 4.3		8	37.6 ± 6.8		8	8.3	45.5	3.8
5th trial	4	8.8 ± .6*		4	10.9 ± 4.8		3	−25.4 ±12.8		4	26.9 ± 4.2**		4	5.8	56.6	3.3
Average		66.7 ± 2.7***			− 4.0 ± 1.2			− 9.8 ± 2.2			35.0 ± 4.1***			7.0	58.9	4.1
Lot VI. Medium Plane of Nutrition, Low Protein, Low Mineral																
1st trial	8	73.3 ± 5.6		8	− 5.8 ± 2.2		8	− 4.3 ± 4.5		7	92.3 ±13.7		8	7.6	57.1	4.3
2nd trial	8	47.4 ± 4.4		8	.9 ± 1.9		8	−10.8 ± 3.3		8	11.8 ± 6.0		8	7.3	63.9	4.7
3rd trial	8	61.9 ± 4.0		8	− 2.7 ± 2.8		8	− 2.5 ± 4.5		8	33.3 ± 4.6		8	6.9	70.1	4.8
4th trial	8	123.8 ± 7.1		8	−11.1 ± 2.2		8	−20.3 ± 4.3		8	33.5 ± 6.8		8	9.9	46.4	4.6
5th trial	4	10.2 ± .6*		4	9.4 ± 4.8		4	37.4 ±11.1		4	36.9 ± 4.2**		4	7.8	52.1	4.0
Average		76.6 ± 2.7***			− 3.1 ± 1.2			− 4.3 ± 2.2			41.1 ± 4.1***			7.9	58.6	4.5

* *Average fibre length in cm. at end of 5th trial.*
** *Average percentage grease to clean wool in fleeces at end of 5th trial.*
*** *Average of first 4 years.*

TABLE III.—*Concluded*

AVERAGE PERCENTAGE INCREASES IN FIBRE LENGTH, FIBRE DIAMETER, TENSILE STRENGTH, GREASE, AND AVERAGE FLEECE YIELDS FOR THE DIFFERENT LOTS DURING THE FIVE YEARLY TRIALS—*Concluded*

—	No. ewes	Increase in fibre length, %	SE	No. ewes	Increase in fibre diameter, %	SE	No. ewes	Increase in tensile strength, %	SE	No. ewes	Per cent increase in % grease	SE	No. ewes	Raw wool prod., lb.	Clean wool, %	Clean wool prod., lb.
Lot VII. High Plane of Nutrition																
1st trial	8	73.3 ± 5.6		8	.4 ± 2.2		8	− 5.6 ± 4.5		7	66.5 ±13.7		7	6.9	58.7	4.1
2nd trial	8	44.1 ± 4.4		8	.5 ± 1.9		8	− 9.9 ± 3.3		8	18.5 ± 6.0		7	8.1	63.3	5.2
3rd trial	8	55.1 ± 4.0		8	− 1.4 ± 2.8		8	− 4.5 ± 4.5		8	39.0 ± 4.6		8	7.3	68.4	5.0
4th trial	8	123.9 ± 7.1		8	− 2.1 ± 2.2		8	− 1.3 ± 4.3		7	40.1 ± 7.3		8	11.4	44.5	5.0
5th trial	7	10.8 ± .5*		7	− 7.5 ± 3.6		7	− 9.2 ± 8.4		6	41.3 ± 3.4**		6	8.4	51.3	4.3
Average		74.1 ± 2.7***			− 1.9 ± 1.1			− 6.0 ± 2.3			40.2 ± 4.2***			8.5	57.4	4.8
Lot VIII. Medium Plane of Nutrition, High Protein																
1st trial	8	79.0 ± 5.6		8	− .9 ± 2.2		8	− 7.1 ± 4.5		7	70.9 ±13.7		8	7.5	59.1	4.4
2nd trial	8	44.0 ± 4.4		8	− 3.3 ± 1.9		8	− 5.4 ± 3.3		8	17.6 ± 6.0		8	8.3	61.1	5.1
3rd trial	8	55.6 ± 4.0		8	− 4.5 ± 2.8		8	−15.4 ± 4.5		8	27.8 ± 4.6		8	6.5	67.0	4.4
4th trial	8	134.5 ± 7.1		8	− 8.3 ± 2.2		8	−16.5 ± 4.3		8	33.1 ± 6.8		8	11.2	45.6	5.1
5th trial	6	9.2 ± .5*		6	−12.4 ± 3.9		4	−28.3 ±11.1		4	53.5 ± 4.2**		4	8.0	46.4	3.7
Average		78.3 ± 2.7***			− 5.5 ± 1.1			−13.0 ± 2.2			36.3 ± 4.1***			8.1	56.9	4.5
Lot IX. Medium Plane of Nutrition, High Minerals																
1st trial	8	78.7 ± 5.6		8	− 3.2 ± 2.2		8	− 1.3 ± 4.5		7	68.3 ±13.7		8	6.4	60.4	3.8
2nd trial	8	40.9 ± 4.4		8	− 2.1 ± 1.9		8	− 8.4 ± 3.3		8	26.0 ± 6.0		8	7.9	64.2	5.1
3rd trial	8	49.4 ± 4.0		8	− 2.4 ± 2.8		8	− 7.1 ± 4.5		8	35.8 ± 4.6		8	6.6	66.6	4.4
4th trial	8	139.3 ± 7.1		8	− 9.1 ± 2.2		8	−18.0 ± 4.3		8	39.2 ± 6.8		8	9.7	43.7	4.2
5th trial	6	10.1 ± .5*		7	− 5.4 ± 3.6		5	−17.9 ± 9.9		5	42.2 ± 3.7**		5	7.8	49.4	3.9
Average		77.1 ± 2.7***			− 4.4 ± 1.1			− 9.9 ± 2.2			41.5 ± 4.1***			7.7	57.4	4.3

* *Average fibre length in cm. at end of 5th trial.*
** *Average percentage grease to clean wool in fleeces at end of 5th trial.*
*** *Average of first 4 years.*

TABLE IV

DIFFERENCES IN AVERAGE PERCENTAGE INCREASES IN FIBRE LENGTH, FIBRE DIAMETER, TENSILE STRENGTH, GREASE AND FLEECE YIELDS BETWEEN TWO GROUPS OF SHEEP MAINTAINED UNDER DIFFERENT EXPERIMENTAL CONDITIONS

—	Diff. in % fibre length increase	SE	Diff. in % increase in fibre diameter	SE	Diff. in % increase in tensile strength	SE	Diff. in % change in % grease	SE	Diff. in raw wool produced, lb.	Diff. in % clean wool	Diff. in clean wool produced, lb.
Effect of High Humidity. Low *vs.* High. Lots II *vs.* I.											
1st trial	− 9.3	± 7.9	− 2.3	± 3.1	+ 3.3	± 6.6	+15.0	±20.1	− .9	− 2.0	− .7
2nd trial	− 9.4	± 6.2	+ 2.5	± 2.7	+10.1	± 4.7	−13.8	± 9.2	+ .6	− 3.0	+ .1
3rd trial	+ 1.0	± 6.1	− 2.4	± 4.1	− 3.0	± 6.6	+ 2.7	± 6.7	− .2	− .3	− .1
4th trial	− 1.7	±10.4	− 3.0	± 3.4	+ 2.0	± 6.6	−17.8	± 9.6	− .4	− 3.1	− .4
5th trial	− 4.3	± 5.1	− .6	± 6.4	−22.3	±14.9	−13.3	±19.9	− .5	− 3.9	− .7
Average	− 6.2	± 3.5	− .5	± 1.7	+ .3	± 3.3	− 5.1	± 5.9	− .2	− 2.6	− .3
Effect of Temperature. Low *vs.* High. Lots III *vs.* I.											
1st trial	−30.7	± 7.9	− 9.8	± 3.1	+ .4	± 6.6	+28.0	±21.0	− .1	− 5.4	− .4
2nd trial	− 3.2	± 6.2	+ 5.0	± 2.7	+ 9.1	± 4.7	+16.1	± 8.5	+ .9	− 4.4	+ .2
3rd trial	− 1.1	± 6.1	− 1.3	± 4.1	− 8.4	± 6.6	− 9.8	± 6.7	− .3	+ .1	− .1
4th trial	−14.9	±10.4	− 2.4	± 3.4	− .7	± 6.6	−12.4	± 9.6	+ .4	− 4.0	− .1
5th trial	+ 1.1	± 5.1	+ .6	± 6.4	−24.5	±14.9	− 7.6	±19.9	+ .4	− 3.1	− .1
Average	−12.4	± 3.5	− 1.1	± 1.7	− 2.6	± 3.3	+ 3.8	± 5.8	+ .3	− 3.7	− .1
Effect of Early Shearing. Late *vs.* Early. Lots IV *vs.* III.											
1st trial	−32.3	± 8.1	+ 2.1	± 3.3	+ 6.4	± 6.6	−15.3	±21.8		+14.7	
2nd trial	−27.0	± 6.4	− .4	± 2.8	+20.0	± 4.8	− 8.8	± 8.8		+ .7	
3rd trial	−40.0	± 5.8	+ 1.7	± 4.1	+ 6.5	± 6.6	+12.0	± 7.0		+ 1.1	
4th trial	−35.1	±10.0	+ 6.0	± 3.1	− 1.8	± 6.0	−13.7	± 9.6		+ 3.7	
5th trial	− .8	± .8*	+ 9.1	± 6.0	−15.5	±13.9	− 2.3	± 5.9**	−2.2	+ .6	−1.2
Average	−34.5	± 4.0***	+ 3.6	± 1.7	+ 7.6	± 3.3	− 6.7	± 6.1***		+ 4.8	

* *Difference between average fibre length in cm.*
** *Difference between average percentage grease.*
*** *Difference between first 4 years averages.*

TABLE IV—*Continued*

DIFFERENCES IN AVERAGE PERCENTAGE INCREASES IN FIBRE LENGTH, FIBRE DIAMETER, TENSILE STRENGTH, GREASE AND FLEECE YIELDS BETWEEN TWO GROUPS OF SHEEP MAINTAINED UNDER DIFFERENT EXPERIMENTAL CONDITIONS—*Continued*

—	Diff. in % fibre length increase	SE	Diff. in % increase in fibre diameter	SE	Diff. in % increase in tensile strength	SE	Diff. in % change in % grease	SE	Diff. in raw wool produced, lb.	Diff. in % clean wool	Diff. in clean wool produced, lb.
Effect of Plane of Nutrition. Low *vs.* Medium. Lots V *vs.* VI.											
1st trial	+ 6.0	± 8.1	− 4.4	± 3.1	−11.5	± 6.3	+42.8	±20.1	+ .5	− .5	+ .2
2nd trial	+ 5.4	± 6.2	+ 2.2	± 2.8	+ 1.9	± 4.7	− 2.9	± 8.5	0	− .1	+ .1
3rd trial	+ 1.3	± 5.7	+ 3.4	± 4.0	0	± 6.3	− 6.5	± 6.5	+ .9	+ .6	+ .6
4th trial	+26.8	±10.0	+ 3.0	± 3.1	+ 4.9	± 6.0	− 4.1	± 9.6	+1.6	+ .9	+ .8
5th trial	+ 1.4	± .8*	− 1.5	± 6.8	+62.8	±16.9	+10.0	± 5.9**	+2.0	− 4.5	+ .7
Average	+ 9.9	± 3.8***	+ .9	± 1.7	+ 5.5	± 3.1	+ 6.1	± 5.8***	+ .9	− .3	+ .4
Effect of Plane of Nutrition. Low *vs.* High. Lots V *vs.* VII.											
1st trial	+ 6.0	± 8.1	+ 1.8	± 3.1	−12.8	± 6.3	+17.0	±20.1	− .2	+ 1.1	0
2nd trial	+ 2.1	± 6.2	+ 1.8	± 2.8	+ 2.8	± 4.7	+ 3.8	± 8.5	+ .8	− .7	+ .6
3rd trial	− 5.5	± 5.7	+ 4.7	± 4.0	− 2.0	± 6.3	− .8	± 6.5	+1.3	− 1.1	+ .8
4th trial	+26.9	±10.0	+12.0	± 3.1	+23.9	± 6.0	+ 2.5	± 9.9	+3.1	+ 1.0	+1.2
5th trial	+ 2.0	± .8*	−18.4	± 6.0	+16.2	±15.3	+14.4	± 5.4**	+2.6	− 5.3	+1.0
Average	+ 7.4	± 3.8***	+ 2.1	± 1.6	+ 3.8	± 3.2	+ 5.2	± 5.9***	+1.5	− 1.5	+ .7
Effect of Plane of Nutrition. Medium *vs.* High. Lots VI *vs.* VII.											
1st trial	0	± 7.9	+ 6.2	± 3.1	− 1.3	± 6.3	−25.8	±20.1	− .7	+ 1.6	− .2
2nd trial	− 3.3	± 6.2	− .4	± 2.7	+ .9	± 4.7	+ 6.7	± 8.5	+ .8	− .6	+ .5
3rd trial	− 6.8	± 5.7	+ 1.3	± 4.0	− 2.0	± 6.3	+ 5.7	± 6.5	+ .4	− 1.7	+ .2
4th trial	+ .1	±10.0	+ 9.0	± 3.1	+19.0	± 6.0	+ 6.6	± 9.9	+1.5	− 1.9	+ .4
5th trial	+ .6	± .8*	−16.9	± 6.0	−46.6	±13.9	+ 4.4	± 5.4**	+ .6	− .8	+ .3
Average	− 2.5	± 3.8***	+ 1.2	± 1.6	− 1.7	± 3.2	− .9	± 5.9***	+ .6	− 1.2	+ .3

* *Difference between average fibre length in cm.*
** *Difference between average percentage grease.*
*** *Difference between first 4 years averages.*

TABLE IV—*Concluded*

DIFFERENCES IN AVERAGE PERCENTAGE INCREASES IN FIBRE LENGTH, FIBRE DIAMETER, TENSILE STRENGTH, GREASE AND FLEECE YIELDS BETWEEN TWO GROUPS OF SHEEP MAINTAINED UNDER DIFFERENT EXPERIMENTAL CONDITIONS—*Concluded*

—	Diff. in % fibre length increase	SE	Diff. in % increase in fibre diameter	SE	Diff. in % increase in tensile strength	SE	Diff. in % change in % grease	SE	Diff. in raw wool produced, lb.	Diff. in % clean wool	Diff. in clean wool produced, lb.
Effect of Protein. Low *vs.* High. Lots VI *vs.* VIII.											
1st trial	+ 5.7	± 7.9	+ 4.9	± 3.1	− 2.[illegible]	± 6.3	−21.4	±20.1	−1.2	+ 3.3	− .5
2nd trial	− 3.4	± 6.2	− 4.2	± 2.7	[illegible]	± 4.7	+ 5.8	± 8.5	+1.0	− 2.8	+ .4
3rd trial	− 6.3	± 5.7	− 1.8	± 4.0	[illegible]2.9	± 6.3	− 5.5	± 6.5	− .4	− 3.1	− .4
4th trial	+10.7	±10.0	+ 2.8	± [illegible]1	+ 3.[illegible]	± 6.0	− .4	± 9.6	+1.3	− .8	+ .5
5th trial	− 1.0	± .8*	−21.8	± 6.2	−65.[illegible]	±15.7	+16.6	± 5.9**	+ .2	− 5.7	− .3
Average	+ 1.7	± 3.8***	− 2.4	± 1.6	− 8.7	± 3.1	− 4.8	± 5.8***	+ .2	− 1.7	0
Effect of Minerals. Low *vs.* High. Lots VI *vs.* IX.											
1st trial	+ 5.4	± 7.9	+ 2.6	± 3.1	+ 3.0	± 6.3	−24.0	±20.1	−1.2	+ 3.3	− .5
2nd trial	− 6.5	± 6.2	− 3.0	± 2.7	+ 2.4	± 4.7	+14.2	± 8.5	+ .6	+ .3	+ .4
3rd trial	−12.5	± 5.7	+ .3	± 4.0	− 4.6	± 6.3	+ 2.5	± 6.5	− .3	− 3.5	− .4
4th trial	+15.5	±10.0	+ 2.0	± 3.1	+ 2.3	± 6.0	+ 5.7	± 9.6	− .2	− 2.7	− .4
5th trial	− .1	± .8*	−14.8	± 6.0	−55.3	±14.9	+ 5.3	± 5.6**	0	− 2.7	− .1
Average	+ .5	± 3.8***	− 1.3	± 1.6	− 5.6	± 3.1	+ .4	± 5.8***	− .2	− 1.2	− .2

* *Difference between average fibre length in cm.*
** *Difference between average percentage grease.*
*** *Difference between first 4 years averages.*

The standard error of the mean (SE_M) for the 97% and 123.8% for the above lots was obtained by dividing the standard deviation for the entire 9 lots by the square root of the number of individuals in each lot or $\frac{\sigma}{\sqrt{n}}$.

The standard deviation for the 9 lots was secured by totalling (as shown above) the squares of the deviations from the mean, d^2, of the 9 lots, dividing such total by the total number of sheep in the 9 lots less one for each lot, and taking the square root of the quotient, *i.e.*,

$$\sigma = \sqrt{\frac{\Sigma d^2}{n - 9}}.$$

The SE_M of 97% and 123.9% was ± 7.1 for each number, as σ and $\sqrt{n}$ were the same for both lots.

In Table III the percentage increases in fibre length for each year are shown with their respective SE_M. Below there is shown a weighted average for the five years; its SE was calculated according to the following equation:

$$SE = \sqrt{\frac{\Sigma(n \times SE_M{}^2)}{Nn}}, \text{ where N is the number of trials.}$$

Thus, for Lots V and VI for the four trials the percentage increase in fibre length was $66.7 \pm 2.7\%$ and $76.6 \pm 2.7\%$.

In Table IV differences between comparable lots are shown for each year and for the five trials combined.

Comparing Lots V and VI to note the "Effect of Plane of Nutrition" in the 4th trial, the figures are 26.9 ± 10. This is merely the difference between 97% and 123.9% (Table III). Its standard error is $\sqrt{7.1^2 + 7.1^2}$.

The same method was used to calculate the average difference in percentage increase in fibre length between Lots V and VI for the five trials. Table IV shows $9.9 \pm 3.8\%$, which is the difference between 66.7 and 76.6 in Table III, with the SE equal to $\sqrt{2.7^2 + 2.7^2}$.

References

1. SACKVILLE, J. P. and BOWSTEAD, J. E. Can. J. Research, C, 16 : 153-165. 1938.
2. LAROSE, P. and TWEEDIE, A. S. Can. J. Research, C, 16 : 166-173. 1938.